THE APPLICATION OF A DIHYDROPYRIDINE-PYRIDINIUM SALT REDOX SYSTEM TO DRUG DELIVERY TO THE BRAIN

BY

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TO MOTHER

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This work has been concerned with the design and testing of a broadly applicable drug delivery system which is specific for the brain. The method developed for this purpose is based on a dihydropyridine⇄pyridinium salt redox system and on the blood-brain barrier. In the proposed delivery system, a pharmacologically active agent which contains a pyridinium nucleus would be reduced to its corresponding dihydropyridine. After systemic administration, the highly lipoidal dihydropyridine would partition into the brain as well as into the periphery. In both locations oxidation would occur. In the systemic circulation, the charge species would be rapidly eliminated by renal or biliary mechanisms while in the brain the compound would be retained.

The prototype compound which was chosen for inclusion in this scheme was berberine. This alkaloid has a high *in vitro* activity against various cancer systems but its *in vivo* activity is low. The first step in the application of the described delivery system to berberine is the synthesis

of dihydroberberine which was accomplished using sodium borohydride in pyridine. The dihydroberberine was analyzed by various spectroscopic means and a number of physical properties were measured. Theoretical calculations using a MINDO/3 approach were also undertaken.

In order to verify the proposed scheme, the delivery of berberine to the brain and the retention of berberine in the brain had to be shown. When dihydroberberine or its hydrochloride salt were injected systemically, high levels of berberine were found in the brain and its efflux from the brain was slow. If, however, berberine is injected systemically, no detectable levels are found in the brain. When dihydroberberine is slowly infused the concentration of berberine rises in the brain and at forty-five minutes, this concentration is specifically higher in the brain than in any other organ analyzed. The mechanism of efflux of berberine from the brain was investigated and appears to be mediated by a passive process, perhaps the bulk flow of cerebral spinal fluid. Dihydroberberine was found to be less toxic than berberine. Preliminary anticancer data indicate that dihydroberberine is more effective in increasing the life span of animals injected intercerebrally with P388 lymphocytic leukemia than is berberine.

CHAPTER 1

INTRODUCTION

A method for delivering drugs specifically to a particular organ would be valuable. Properly designed, a drug delivery system should concentrate an agent at its site of action and reduce its concentration in other locations. The results of these manipulations would not only be an increase in the efficacy of an agent, but also a decrease in its toxicity.

A site specific system designed for the central nervous system (CNS) would be especially useful. The reason for this, aside from the inherent importance of the brain, is that the entry of many pharmacologically active agents into the CNS is impeded by a set of specialized barriers present at the blood-brain interface. This barrier system, termed the blood-brain barrier (BBB), is composed of numerous enzymatic and anatomical components.

This dissertation will present a general method for the specific delivery of drugs to the brain and give an example which substantiates the method. In order to provide an adequate background for a discussion of drug delivery to the brain, a review of the BBB is necessary. The introductory material is then continued with a cursory historical account of drug delivery systems and prodrugs. Because of the importance and great interest of anticancer agents, emphasis is placed on this topic in this section. The closing section of the first chapter will state specific aspirations as they apply to the present research.

Blood-Brain Barrier

Structural and Enzymatic Considerations of the Blood-Brain Barrier

The existence of a barrier which separates central nervous tissue from the general circulation was first postulated by Ehrlich at the end of the 19th century.[1,2,3,4,5] In a series of pioneering experiments, he injected a number of dyes into laboratory animals and found that, while the visceral organs were highly stained, the brain was conspicuously uncolored. It was later discovered that these dyes bind extensively to plasma proteins so that the actual barrier then described was one to these complexes. Many small hydrophilic compounds cannot, however, pass into the brain so that this barrier is presented to a wide variety of compounds. Early in the study of the BBB, the obstruction was considered absolute but this idea was soon dispensed with since the nutritional requirements of the brain necessitate the equilibration of a number of compounds between the general circulation and the CNS.[3]

The morphological basis of the BBB had been a very controversial subject until relatively recently. Historically, three hypotheses have been put forward to explain this impermeability to blood-borne substances.[1] All are based on structural differences between the cerebral vascular system and the systemic circulation. It was proposed that the small extracellular space characteristic of mammalian brains prohibited the accumulation of compounds and, as such, constituted a barrier. It was shown, however, that some animals with large extracellular spaces have a well-defined barrier to a number of substances.[6] The suggestion was also made that the general impermeability of the brain to blood-borne substances was due to astrocytic end feet which surround the capillaries, forming an envelope, or due to the endothelial cell lining of the cerebral capillaries.[1] In order to study this question, electron microscopic evaluation is necessary.

Progress was somewhat slowed because of the lack of appropriate electron microscopic tracers.[7] The first tracers used were saccharated iron oxides[8] or ferritin (molecular weight 560,000) whose limits of resolution were close to the thickness of the endothelium itself. It was not until the introduction of horseradish peroxidase (HRP) and microperoxidase (MP) that the exact structure of the BBB could be deduced. Horseradish peroxidase is a relatively small enzyme (molecular weight 43,000) which, unlike ferritin, does not contain an electron-dense core but rather produces a material which has a high affinity for osmium tetraoxide and other radiopaque substances.[7]

By using HRP, Reese and Karnovsky demonstrated the inability of the marker to pass from the lumen of the cerebral capillary.[9,10] In fact, HRP was never found in the extracellular space surrounding the capillary. Additionally, when HRP was injected directly into the brain it readily passed the astrocytic end processes and was stopped at the endothelial membrane. The anatomical basis of the BBB was, therefore, isolated to the endothelial lining of the cerebral capillaries and not a perivascular site. There are several ultrastructural differences between systemic capillaries and cerebral capillaries which account for their general impermeability.[1,4]

The manner in which endothelial cells of the cerebral capillaries are joined is distinct from systemic capillaries. Cerebral junctions are characterized as tight or closed junctions meaning the cells closely approximate each other. These junctions gird the cell circumferentially, forming a zona occludens and providing an absolute barrier to HRP. Structurally, the junctions consist of aligned intramembranous ridges and grooves which are in close apposition.[11] These tight junctions have been examined by thin section electron microscopy and attempts are now underway to examine them by a freeze-fracture technique.[4,12] This method, which allows a longitudinal

view of the capillaries, will add greatly to the structural knowledge of these tight junctions but the method is technically difficult. Recently, a freeze-fracture technique was applied to the cerebral vasculature of a chameleon, which possesses a BBB similar to that of mammals.[13] In these animals a series of ridges and grooves is seen. The ridges are connected to neighboring ridges by an anastomosing network. In general, the more complex this system is, i.e. the number of ridges it has, the tighter the junction is. The tightness of the junction can be assayed not only structurally but also by measuring ionic conductance and resistance through the junction. The ionic conductance is low for most ions.[12,14]

Systemic capillaries lack this closed junction. Morphologically, this can be traced to a lack of continuity in the intercellular appositions. In cardiac muscle, for example, the ratio of junctional width to the width of the cell membrane is 2.4, while in cerebral capillaries this is reduced to 1.7.[10] These open junctions allow a high degree of nonspecific transport of nutrients and other compounds into systemic capillaries. Materials pass easily between these leaky cells, while in the brain the sealing of the intercellular fissures severely restricts this nonspecific transport. Since intercellular transport is removed, only intracellular transport remains. Lipophilic compounds can readily pass through these phospholipoidal membranes, but hydrophilic compounds and compounds with high molecular weights are excluded. Systems are available to transport small hydrophilic nutrients, and this will be discussed later.

A second difference between cerebral and systemic capillaries is the paucity of vesicles and vesicular transport in the CNS.[4,7] Vesicular transport is a process for transcellular transport and, as such, vesicles are transported from the luminal to the abluminal membrane. Pinocytotic activity, on the other hand, is concerned with the nutritional requirements of

the cell and, therefore, involves vesicular movement from the luminal membrane to a cell organelle, presumably a lysosome.

Cerebral endothelial vesicles are usually uncoated and few in number compared to other systems. Using electron microscopic morphometry, five vesicles per micrometer luminally and thirty-forty vesicles per micrometer abluminally were found.[4] In the diaphragm and in myocardial vessels the values are much higher, being seventy-eight and eighty-nine vesicles per micrometer, respectively. This lower content of vesicles is another mechanism by which the CNS can limit nonspecific influx. A third difference is the lack of fenestra in the cerebral capillaries. These differences are demonstrated schematically in Figure 1-1.

Cerebral vessels have a number of perivascular accessory structures which appear to be involved in BBB function.[4] While it is known that the astrocytic end feet are not involved as a barrier per se, their role in attenuating BBB action is interesting. These glial end feet may be involved with regulation of amino acid flux. They also appear to engulf protein which breeches the BBB and, therefore, may act as a second line of defense.[15] Phagocytic pericytes which are present abluminally may play a similar role. It is possible that the basement member of endothelial capillaries acts as a mass filter preventing large molecules from penetrating it.

In addition to these structural features the BBB maintains a number of enzymes which appear to augment barrier function.[2,16,17] Since optimal neuronal control requires a careful balancing between neurotransmitter release, metabolism, and uptake, it is of vital importance to restrict the entry of blood-borne neurotransmitters into the CNS. It is not surprising, therefore, to find high concentrations of such enzymes as catechol-O-methyl transferase (COMT), monoamine oxidase (MAO), γ-aminobutyric acid transaminase (GABA-T) and aromatic amino acid decarboxylase (DOPA decarboxylase)

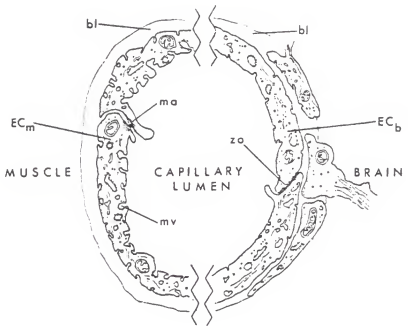


Figure 1-1. This Schematic Illustration Represents an Endothelial Cell Derived from either a Muscle (EC_m) or Brain (EC_b) Capillary. In this Figure, (ma) is the Macula Adherens or Loose Junction, (zo) is the Zona Occludens or Tight Junction, (mv) are Microvesicles, and (bl) is the Basal Lamina. This Figure was Modified from Reference 1, Page 162 by Permission.

in the BBB. Recently, a distributional study of COMT in the brain indicated that this enzyme is present in many sites including several which lack a structural BBB.[18] This distribution may aid in the exclusion of neurotransmitters from structurally unprotected areas. The presence of DOPA decarboxylase explains partially the need for giving such large doses of L-dihydroxyphenylalanine (DOPA) in the treatment of CNS dopamine deficiencies to achieve appropriate therapeutic cerebral levels. The enzymatic BBB may also play a role in the exclusion of lipophilic compounds which might otherwise passively diffuse through the BBB. This is suggested by the presence of pseudo (butyryl) cholinesterase in the cerebral capillaries.[4] This enzyme is not found in noncerebral capillaries. The occurrence of γ-glutamyltranspeptidase has also been described and may account for some protection from peptide infiltration.[2,19] An early proposal that γ-glutamyltranspeptidase is involved with carrier systems is questionable. Acid phosphatase activity, which is a marker for lysosomes and pre-lysosomes or phagosomes, is present in the endothelial cells.[15] These organelles appear to be involved in the degradation of endocytosed material and, as such, can be considered a component of the BBB. The endothelial cells of the cerebral microcirculation contain a large number of enzymes but care must be taken in ascribing a certain enzyme to a barrier role.[17] There are numerous enzymes which, while present in the endothelial cells, do not serve in any capacity other than general cellular functioning.

Molecular Carriers Involved in BBB Transport

The aspects of the BBB which have been discussed thus far give an indication of its relative impermeability to a number of blood-borne substances, but do not explain the movement of essential nutrients into the CNS. This transport is brought about by a number of carriers which are situated in the endothelial cells. These carriers are generally assumed to be proteinaceous.

They are equilibrative, i.e. nonenergy dependent and bidirectional in nature and can be saturated.[1,20,21] The net movement of compounds is always along a concentration gradient and since nutrients are readily utilized as soon as they pass into the brain, this gradient is in the direction of the brain.

A number of specific carriers for compounds have been described. The first to be characterized was one for hexoses.[1,12,22] This carrier displays saturable kinetics and can be competitively inhibited. The hexose carrier is stereospecific and has a high affinity for α-D-glucose with a Km between 6.0 and 9.0 mM. Other sugars with affinity for this carrier include, in order of decreasing affinity, 2-deoxy-D-glucose, 3-O-methylglucose, β-D-glucose, D-mannose, D-galactose and D-xylose.[22] The Km of D-fructose and L-glucose is very high.

The carrier is Na^+ independent and is inhibited by phloretin, a non-competitive inhibitor, more than phlorizin, a competitive inhibitor.[20] The V_{max} is similar for all sugars tested which indicates the rate limiting step for transport is not the association of the sugar with the carrier but, rather, the movement of the carrier across the membrane complex. This carrier demonstrates exchange diffusion, i.e. the carrier moves more rapidly when loaded than when empty.[22,23]

At a Km of 7 mM, the concentration of glucose required to produce saturation is about 126 mg% so that under physiological conditions, the system is about half saturated. In normal situations the rate determining step in glucose utilization is the hexokinase step. This can be shifted to BBB transport of glucose in conditions of hypoglycemia.[21] While substrate flux is usually thought of as being related only to plasma levels of glucose, recent studies have indicated that intracerebral glucose concentrations can alter carrier kinetics.

The effects of insulin on the transport of glucose are controversial.[22,24] Several reports have indicated no effect on either unidirectional or net flux after insulin infusion. This is curious in that insulin would not be expected to pass the BBB. The presence of insulin receptors in cerebral capillaries may explain this enigma in that insulin may bind to a receptor on the luminal surface and its effects may be mediated to the abluminal surface by a second messenger.[24]

Also controversial is the possibility that a low and high affinity system is operating in glucose transport.[20,22,23] There is a nonspecific flux associated with glucose of 7%. Some authors attribute this to diffusion but an alternate hypothesis has been proposed. This involves the presence of two systems on the carrier: a high affinity, low capacity system and a low affinity, high capacity system. Most data have been collected in isolated vessel preparation but there is some support of this proposal from in vivo experiments. Gjedde showed the putative low affinity system to be stereoselective and to have a Km of 1.0 M,[23] compared with a Km of 1.1 mM for the high affinity system. He contends that a single set of kinetic parameters does not adequately describe the system and that this high-low affinity system better correlates with the data. A suggestion that a protein tetrameter with both low and high affinity sites was the carrier has also been made. At the choriod plexus there appears to be ouiban sensitive, Na^+ dependent glucose flux and this is apparently important in cerebral spinal fluid (CSF) homeostasis.[22]

Three carriers have been described for amino acid transport.[1,12,20] These carriers have affinity for neutral, basic, and acidic amino acids. In general, essential amino acids, which are large and bulky, are transported in preference to nonessential amino acids.[20] In all of these

systems, net flux is small compared to unidirectional flux since amino acids derived from proteins are constantly being lost and the magnitude of this loss is similar to uptake.

The transport of neutral amino acids has been described by Christensen and these generalizations apply to the BBB.[25,26] Four neutral amino acid transport systems have been shown to occur in Ehrlich ascites, a model cell system, and in several other systems. An L- or leucine-preferring system is characterized by high affinity for phenylalanine, leucine, tyrosine, tryptophan and several other large essential amino acids. It is Na^+ independent, bidirectional and equilibrative in nature. The definition of this system can be made by observing the flux of 2-aminonorbornane-2-carboxylic acid which is exclusively transported by this carrier. The A- or alanine-preferring system is characterized by a Na^+ dependence, an energy dependence, and the ability to concentrate substrates. The system has affinity for glycine, proline, alanine, serine, threonine and several other small amino acids. The defining compound for this system is α-(methylamino)-isobutyric acid. Two other amino acid transport carriers have also been described but they are not well characterized. There is also an ASC (alanine-serine-cysteine-preferring) system and a Gly (glycine-preferring) system.

Since generally only large essential amino acids (L-system substrates) are transported through the BBB, the L-system is assumed to be the dominant mechanism in amino acid uptake.[1,20] The absence, however, of the A-system has been questioned. It was recently argued that the A system is present but has a different distribution than the L-system. Betz and Goldstein discovered a system whose characteristics are similar to the A system but which is located abluminally.[27] This system may act as an active mechanism for

efflux of these amino acids from the brain parenchyma or, more probably, for concentrating them in the endothelial cytosol. The necessity for this concentration is related to a proposal that the L and A systems may act together in the transport of amino acids. This hypothesis is based on a possible equilibration of amino acids between the two carriers in the cytoplasm of the endothelial cell.[26]

In many cases the Km value for an amino acid is similar to its plasma concentrations. This being the case, slight changes in the blood levels of an amino acid may alter their disposition. The utilization of tryptophan, for example, which is a precursor of serotonin, is partially determined by its availability and its movement across the BBB.[21,28] A similar situation may exist in certain circumstances with tyrosine, which is the precursor for dopamine, norepinephrine and epinephrine.

Recently, a hypothesis was forwarded to explain the regulation and induction of these carriers.[29,30,31] It has been suggested that an amino acid is produced abluminally in large amounts and transported on the neutral amino acid carrier. The amino acids in such a system should have a high Km and, therefore, be easily displaced from the carrier at the luminal side or in transit. These carriers, like the hexose carriers, exhibit exchange diffusion. The proposed amino acid in this regulatory role is glutamine. Glutamine is synthesized from glutamic acid and ammonia by glutamine synthetase in astrocytes, a perivascular locus. The concentration of glutamine is ten times higher than the concentration of any other neutral amino acid and its local concentration in the vicinity of the BBB is predicted to be still higher.[30]

This hypothesis was formulated on the basis of several interesting observations. In cases where ammonia and, presumably, glutamine levels

increase cerebrally, as in porto-systemic shunts, the uptake of phenylalanine and other neutral amino acids increases. Also, if glutamine synthetase is inhibited by methionine sulfoximine, the uptake of essential amino acids is reduced.[29] While this is an attractive proposal, several authors have questioned it on the grounds that many amino acids compete for this neutral amino acid carrier and the role of glutamine, therefore, may be relatively unimportant.[32]

Relatively little work has been done with basic and acidic amino acids. The acidic system has affinity for glutamate and is fully saturated at physiological concentrations. Basic amino acids are transported on a distinct carrier. This carrier is equilibrative, saturable and bidirectional and has been described as a Ly^+ or lysine-preferring system.

A monocarboxylic acid carrier has been described which demonstrates affinity for lactate, pyruvate, acetate, propionate, butyrate, δ-aminolevulinic acid and ketone bodies.[1,20] Ketone bodies, such as β-hydroxybutyrate and acetoacetate, are produced in a number of stressful circumstances, including starvation.[33] The carrier possesses similar characteristics to those which have already been described. It is stereospecific and pH sensitive.[20] The transport of the substrates on the carrier increases with a decrease in pH. This has been interpreted to mean either that a proton is cotransported with the acid or that hydroxide acts as a high affinity competitive inhibitor. Only the ionized acid is transported by this system and this has led to the proposal that an $R\text{-}NH_3^+$ moiety is present in the active site. The unionized acid can pass through the membrane by simple diffusion.

The Km of lactate is similar to the plasma level of lactate. This being the case, rapid rises in systemic lactate levels, such as the ones which accompany exercise, are not transferred to the brain immediately.[20]

A carrier for choline was described in 1978.[34] Choline cannot be synthesized de novo in the brain but this precursor is required for such important cellular components as acetylcholine and phosphatidylcholine. The carrier conforms generally to those characteristics specified for other systems. It has affinity for choline, hemicholinium, dimethylaminoethanol (deanol), tetraethylammonium, tetramethylammonium, cartitine and spermine but not for NH_4^+. The distribution of this carrier is not uniform. Choline uptake decreases with age and this correlates with an age-dependent diminution of the carrier. The rate determining step in choline utilization is its movement across the BBB.

A carrier with affinity for nucleosides such as adenosine, guanosine and inosine has been described.[1,20] Additionally, a system for transporting purine bases has been isolated. This system transports adenine, guanine and hypoxanthine but not pyrimidines. This is interesting in light of the fact that pyrimidines can be synthesized in the brain from NH_4^+ and aspartate, while purines cannot. This carrier is very active in neonates, but its activity diminishes with age.[35]

Recently, a carrier was described for thyroid hormones.[36,37] The carrier has affinity for triiodotyrosine (T_3) and thyroxine (T_4) but not for tyrosine, leucine or potassium iodide. The transport of T_3 is saturable and inhibited by T_4. The carrier is weakly stereospecific and of high affinity. The presence of a carrier for T_3 and T_4 is interesting because these compounds are fairly lipophilic. The bulkiness of the molecule, however, tends to decrease its ability to pass membranes. A carrier-mediated transport has also been proposed for thiamine.[38] These systems are summarized in Table 1-1.

Table 1-1. Blood-Brain Barrier Transport Systems

Transport system	Representative Substrate	Km (mM)	V_{max} (nmol $min^{-1}g^{-1}$)
Hexose	Glucose	9	1600
Neutral Amino Acid	Phenylalanine	0.12	30
Acidic Amino Acid	Glutamate	——	——
Basic Amino Acid	Lysine	0.10	6
Monocarboxylic Acid	Lactate	1.9	120
Amine	Choline	0.22	6
Nucleoside	Adenosine	0.018	0.7
Purine	Adenine	0.027	1
Thyroid Hormone	T_3	0.001	0.17
Thiamine	Thiamine	——	——

In addition to these carrier systems, there are a number of active efflux mechanisms. Two of these systems are located in the choriod plexus and have affinity for organic ions. A system for the disposition of anions has been described and divided into two subsystems.[20,39,40,41,42] An L or liver system with affinity for prostaglandins, 5-hydroxyindole acetic acid and probenicid, as well as a K or kidney system with affinity for p-aminohippuric acid and phenol red, has been proposed. Additionally, a carrier for the efflux of iodide has been described.[43,44] A cationic system is also present in the choriod plexus and this species has affinity for N-methylnicotinamide, decamethonium and hexamethonium ions.[45,46] These systems appear to be important in the removal of metabolic acids and bases. This, as well as the anionic system, is energy-dependent and can be competitively inhibited. The presence of several energy (ATP)-dependent systems in the capillaries indicates that this site may also be important for active efflux.[19] The high density of mitochondria in cerebral capillaries is further support for this location.[47]

Movement of Compounds Across the BBB

The BBB, therefore, consists of a relatively impermeable membrane superimposed on which are mechanisms for allowing the entrance of essential nutrients and the exit of metabolic wastes. If a compound is to gain access to brain parenchyma, it may do so via several routes. If the agent has affinity for one of the carriers previously described, it may diffuse across the BBB by association with this carrier. A compound which has a high intrinsic lipophilicity can diffuse passively through the phospholipoidal cell membrane matrix.[1,4,12] The pK of a compound with ionizable groups is also important, since only the unionized species diffuses across the BBB rapidly.[48,49] The ability of a substance to enter into the cell membrane

is often correlated with its in vitro octanol:water partition coefficient.[50,51] This is a measure of lipophilicity, and can be correlated with biological effects.[52] These correlations can be extended to the permeability of compounds through the BBB. Several examples of this correlation have appeared in the literature, including the opiates morphine, codeine, and heroin.[52] Good correlation is obtained between lipophilicity, the ability to pass the BBB, and narcotic efficacy.

The increase in the ability of a compound to pass membranes can, all too often, be correlated with an increase in undesirable side effects. An example of this was shown in a series of β-blockers in which lipophilic members of this pharmacologic group, i.e. propranolol penetrated the BBB rapidly but demonstrated a number of deleterious psychiatric manifestations.[53] Conversely, β-blockers of lower lipophilicity exhibit an attenuation of these side effects.

These two avenues, namely passive diffusion and carrier mediation, represent the major components of influx. Other minor mechanisms may also allow the entry of substrates into the CNS. The cell bodies of many neurons are located centrally while their axons may penetrate into the periphery. These axons can take up material and transport it in a retrograde fashion to the CNS.[7,54] This retrograde axoplasmic transport has been observed in such areas as the nucleus ambiguus and the abducen nucleus. In some cases, however, the endocytosed material is reacted with lysosomes and, therefore, this transport route may have a protective function.

There are several areas of the brain which lack a BBB.[1,8,55] These include such locations near the ventricles as the area postrema, the subfornical organ, the median eminence of the neurohyphosis, the organum vasculosum of the lamina terminalis, and the choriod plexus. Collectively,

these areas are termed the circumventricular organ. In addition, the pineal gland lacks a BBB. These areas constitute a small fraction of the total surface area of the BBB and may allow a limited nonspecific flux.

These loci do have important pharmacological ramifications as demonstrated by the action of a series of atypical neuroleptics.[56,57] These compounds are so named because they provoke certain symptoms of dopaminergic blockade but not others. Specifically, metoclopramide exerts an antiemetic action but not an antischizophrenic effect. This dichotomy was explained by the fact that metoclopramide does not penetrate the BBB. The site of action of antiemetic agents is at the chemoreceptive trigger zone, which is located in the area postrema and is outside the BBB. The apparent inability of metoclopramide to produce the full spectrum of changes which occurs as a consequence of dopaminergic blockade may be related to its pharmacokinetics and, specifically, its inability to pass the BBB. This relegates the compound to only those sites of action not protected by the BBB. Other pharmacologically important sites outside the BBB include the median eminence, which controls prolactin secretion. In some areas, while the BBB is not absent it is diminished. These areas include certain arteriolar segments whose diameters are between 15-30 μm.[4,58,59] In these segments limited protein extravasation, or leakage of compounds from the lumen of the capillary to the extracellular spaces, has been observed.

The ability of small peptides to penetrate the BBB is an extremely controversial point.[21,60,61,62] Systemic administration of certain centrally active peptides elicits a central response. Differences concerning the interpretation of these data are significant. One view is that the flux of these small peptides across the BBB is low indicating, perhaps, some nonspecific route accounts for their entry.[21,62] The presence of peptidyl

receptors luminally, whose stimulation results in the generation of a second messenger, has been proposed in this respect. A different conclusion is that small peptides have a significant flux across the BBB. The peptides which have been investigated thus far include stabilized enkephalins and endorphins, a nonapeptide which induces delta-sleep, melanin stimulating hormone (α-MSH) and melanin inhibiting factor one (MIF-1).[60,61,63]

The BBB plays a major role in CSF homeostasis.[12,64] Cerebral spinal fluid is produced at the choriod plexus and drains from the ventricals through the foramina of Magendie and Luschka into the ventral aspects of the brain.[65] This fluid serves many important mechanical and nutritive functions. Cerebral spinal fluid flow is constant with a $t_{\frac{1}{2}}$ of renewal of about two hundred and seventy minutes. The ventricular volume of CSF is about 23 ml while the subarachnoid volume is 117 ml. Cerebral spinal fluid, along with any dissolved materials, leaves the subarachnoid space via the arachnoid villi, which protrude into a venous sinous. The arachnoid villi act as a one-way valve and prevent backflow.[66] This loss of CSF provides a slow mechanism for nonspecific efflux of compounds from the CNS. This mechanism rids the brain of polar compounds such as metabolic wastes at a fairly constant rate regardless of molecular weight. If a compound is fairly polar and does not have affinity for any passive or active efflux mechanism, it will leave the CNS by CSF bulk flow. Therefore, while lipophilicity is very important for influx to the brain, the efflux of a compound is only partially dependent on this parameter.[46,67,68,69,70]

Since the ionic environment in which neurons function is so important, the composition of the CSF is strictly maintained within narrow limits. The CSF is not simply an ultrafiltrate of plasma, and several concentration gradients are produced. The maintenance of these gradients can, in part, be attributed to the low ionic conductance of the BBB. This is especially

important for K^+, since a low K^+_{csf}/K^+_{plasma} ratio apparently acts to stabilize neurons.[12,71,72]

One of the major factors in influencing diffusion into the CNS is the degree to which a substance is bound to plasma proteins. It had been assumed for a long time that only the free, dialyzable fraction of the total plasma concentration of a compound was available for diffusion.[7] This is important for a great number of compounds. The major species involved in this binding are serum albumins, which tend to bind molecules loosely but to a large extent, and globulins, which bind with high affinity and low capacity. The premise that only the unbound species is capable of diffusion has, however, been challenged.

Steroids have profound central effects and gain entry into the CNS by simple diffusion across the BBB, even though they are highly bound to plasma proteins.[5] This diffusion correlates well with the octanol:water partition coefficient of the steroids and inversely with the tendency of the molecules to form hydrogen bonds.[73] Different steroids are taken up differently, however, and this is in large part related to protein binding. There are several globulins which bind specific steroids. These include sex hormone binding globulin (SHBG), which is found in man but not rats, cortical binding globulin (CBG), estradiol binding globulin (EBG) which, in fetal and neonatal rats, may be synonymous with α-fetoprotein, progesterone binding globulin (PBG), which is found in pregnant guinea pigs and also, thyroid hormone binding globulin (TBG), which is found in man.

Those steroids which are bound to globulins such as cortisone to CBG in rats have a small flux into the brain while those steroids which are bound more highly to albumins such as progesterone, estrogen or testosterone in the rat, easily diffuse through the BBB.[73] The reason for this is that

binding to albumin is sufficiently weak that the capillary transit time in the brain is long enough to allow dissociation of the compound from the macromolecule. In the case of globulins, however, the binding is tighter and the turnover is only of the order of 3-10%/second. The sojourn through the cerebral capillaries is not, therefore, sufficient to release the bound material. It is not, therefore, the plasma-protein-bound fraction which is unavailable for transport but, rather, the globulin-bound fraction.

These principles also apply to free fatty acids such as palmitate. In this case, however, there appear to be high and low affinity sites on the albumin molecule so that the rate of dissociation of the palmitate bound to the site with the lower Km may be slow compared with capillary time, while the low affinity site may not.[74] Melatonin, which is also highly protein bound, shows a significant diffusion through the BBB.[75]

Thyroid hormones which are transported into the CNS by carriers are also bound by plasma proteins.[36,37] The carrier, whose Km is lower than that of the albumin site, is able to compete successfully with the albumin for binding. This is effectively a stripping of the compound from its albumin site. The effect is also seen with tryptophan and other amino acids.[28]

The BBB in Pathological and Experimentally Altered States

The integrity of the BBB is known to be impaired in a number of pathological or experimentally-induced conditions.[4,76,77] The effect produced can be the result of changes of the structural components of cerebral capillaries such as the junction or vesicular activity and, as such, results in generalized increases in permeability. Alternatively, the carrier systems may be compromised and this may lead to specific changes in permeability.

Generalized increases in permeability result in a number of deleterious events. Since the BBB is relatively permeable to water, but not to most other substances, osmotic gradients can be rapidly changed. If plasma proteins and other compounds are allowed to freely enter the CNS, they will bring with them large amounts of water, with the result being cerebral edema.[1,20]

The morphological basis of these changes in any particular situation can be highly controversial. This is especially the case with hypertonic treatment of cerebral capillaries. It has been known for some time that hypertonic solutions of such solutes as glucose, sucrose, urea, arabinose, lactamide and several others can increase the permeability of the cerebral capillaries to protein and other small polar compounds. Rapoport explains this phenomenon as the result of osmotic shrinkage of the endothelial cells, resulting in a pulling apart of the tight junctions.[5,71] This has been challenged. If HRP is injected after a hypertonic solution, the HRP reaction product does not form a continuous line from the luminal to the abluminal surfaces at the junction or at any other location.[4,20] It was suggested, therefore, that increased vesicular transport accounted for the increased permeability, perhaps as a result of increases in local blood pressure.[77]

In any case, if the concentration of the hypertonic solution is close to the critical opening concentration, the opening of the BBB is transient and does not produce acute edema.[5] This procedure may have therapeutic applications. In many cases it is desirable to introduce highly polar compounds into the brain and this osmotic opening of the BBB may provide an avenue for that purpose. Methotrexate is a folate inhibitor used in the treatment of cancer. The pK_a of the carboxylic acid functions of this

molecule is 4.7 and at physiological pH methotrexate is 99.8% ionized and, as such, passes the BBB very slowly, if at all. After pretreatment with hypertonic arabinose, the concentration of methotrexate showed a fifty-fold increase in the CNS.[78]

Hypertension is a major health problem and can produce a number of debilitating complications.[79] Hypertension increases the extravasation of albumin, sucrose, and other polar compounds.[80,81,82] This has been studied in a number of animal models of hypertension including those in which the increased system blood pressure is induced with either amphetamine, ephedrine, Aramine^{R} or bicuculline.

The basis for this increased permeability has been debated frequently and appears to be related to increased vesicular transport since ultrastructural investigations do not indicate capillary lesions or junctional openings.[77] Increased vesicular transport has, in fact, been implicated in a number of situations in which capillary permeability increases. The factors that affect vesicular formation which are described here also apply in those cases.

The mechanism by which hypertension elicits an increase in vesicular activity is not clear. The increased hydrostatic pressure may act to induce an invagination. Also, there are a number of substances associated with hypertension that induce vesicular formation.[4,77,83] These include the catecholamines, serotonin, and histamine. Joo explained the increased vesicular transport in terms of a cyclic 5'-adenine monophosphate (cAMP) stimulation mediated by a catalyzed adenyl cyclase, since cAMP can directly induce vesicular formation.[84,85]

Both serotonin and histamine have been shown to increase protein extravasation and both act to catalyze specific adenyl cyclase. A perivascular

source of both of these compounds is available, since histamine is stored in perivascular mast cells and serotonin in platelets.[4] The action of histamine is partially reversible by H_2 antagonists.[86] Recently, cyclic guanine monophosphate (cGMP) has been isolated in cerebral capillaries and this may play a role in vesicular transport.[87] These effects are seen peripherally as well as centrally.

The major controversy surrounding this area is whether hypertension itself is responsible for extravasation, or if some humoral agent produced as a result of hypertension is culpable. It is difficult to look at this in vivo since many agents can increase blood pressure and increase vesicular transport.

Additional evidence that vesicles are important in extravasation is indicated by the decreased protein flux in capillaries treated with compounds which decrease vesicular formation. These compounds include imidazole, which alters cAMP function by inhibiting the inactivation of phosphodiesterase, thioridazine, a phenothiazine which decreases vesicular fusion with the cell membrane, and desipramine.[80] The anionic transport blocking agent 4-acetamido-4'-isothiocyanostilbene-2,2'-disulfonic acid disodium (SITS) inhibits exocytosis.[88] This compound, which also inhibits protein extravasation in hypertension, inhibits ATP-evoked adrenalin release from chromaffin granules as well as serotonin secretion from platelets. Increased vesicular transport has also been related to the formation of transendothelial channels.[54,89] These channels may be the result of the simultaneous opening of a chain of vesicles and may provide an avenue for nonspecific flux in hypertension. The presence of this channel is highly debated, however.

The nature of the hypertension itself can be a factor in increased capillary permeability.[82] While acute hypertension is very likely to produce a protein influx, chronic hypertension may protect the system in the likelihood of an acute rise in blood pressure. The reason for this is probably the hypertrophy of vascular muscles. This hypertrophy leads to an increased vascular resistance and thickening of the vessel wall providing a mechanism for handling greater pressures. This is consistent with the observation that vasoconstriction decreases capillary permeability, while vasodilation increases this parameter.

In some cases hypertension may lead to the rare malady, hypertensive encephalopathy.[81] This disease is related to the inability of the cerebral vasculature to acclimate to acute severe hypertension. In this instance cerebral edema occurs and this may cause swelling of the nervous tissue. This yields an increased cerebral pressure and may prevent vasodilation caused by a variety of stimuli.[71]

Hypervolemia may also result in an increase in protein extravasation.[90] If mice, whose blood is about 1.3 ml, are treated systemically with high volumes (1 ml) of saline, extravasation of HRP can be documented. At lower volumes, however, no changes in permeability are observed.

Since, in these experiments, the blood pressure increases only by 20 mm Hg, the increased capillary permeability may not be due to hydrostatic pressure. In order to open the BBB, pressure increases on the order of 100 mm Hg are required.

The vasculature of primary brain tumors and of metastatic secondary brain cancers exhibits a generalized degradation.[7,91,92] This breakdown can be characterized by the presence of gap junctions, fenestra and open endothelial junctions, indicating the typical electron microscopic

structure of the tight junctions is destroyed. Although it has been suggested that these deficiencies should render cerebral tumors susceptible to treatment,[93] the clinical evidence does not support this. The therapy which has, thus far, been directed to brain neoplasms has been disappointing at best. Neurosurgery may have reached its practical limit and most agree that major new advances must come from the field of chemotherapy.[91] It is interesting to note that many agents are effective against certain peripheral tumors, but not their secondary brain metastases.

The unresponsiveness of cerebral tumors has been explained by two phenomena. First, it is known that the greatest display of disintegration of the capillary structure occurs in the central, slow growing portion of the tumor, and as one moves to the periphery, the abnormalities decrease.[92] This area of the tumor would be expected to show the highest resistance to chemotherapeutic agents. Secondly, since only a relatively small area of the brain vasculature is disturbed, any drug which reaches the neoplasm would rapidly diffuse into the outlying areas, thereby diminishing its concentration and effectiveness at the tumor site.[78] Few suggestions have been forwarded to circumvent this problem. One involves osmotic opening of the BBB followed by methotrexate treatment, but this has a very limited usefulness because of problems with the accompanying edema.[78]

Many experimental models of brain injury are associated with an increase in capillary permeability.[4,57,94] These models include injury induced by dropped weights, pendulums, hammers, spring-mounted weights, blasting caps, rotary strikers, compressed air guns, humane stunners and accelerating devices. Generally, these procedures have been applied to the cat.

The leakage of proteins from the capillaries is usually proportional to the amount of injury, but even in severe injury the endothelial cell is intact and shows no sign of lesion.[94] The explanation most often proposed for this extravasation is increased vesicular activity. In most injuries there is an increase in serotonin and norepinephrine levels, as well as the production of a number of humoral agents. The increased vesicular content, which appears first in the arterioles and subsequently in the capillaries, may be mediated by stimulation of cAMP. There are also, however, increases in blood pressure and that may act to increase vesicular transport.

Cerebral infarcts also disrupt BBB function, as evidenced by an increased albumin concentration in the CSF of infarct victims.[95] Correlation, however, between infarct size and location and the quantity of albumin leaked has not revealed any significance.

Both electroconvulsive shock and pentylenetetrazole-induced seizures result in an increased capillary permeability to a number of plasma markers.[1,4,54] The degree of permeability increase is proportional to the number of shocks or compounds given. The morphological basis for this extravasation is not known and both junctional opening and increased vesicular transport have been suggested.[77] It is interesting to note that, in both of these procedures, the blood pressure rises and this may be an important factor.

Induced ischemia also produces a marked increase in the permeability of the cerebral vasculature.[54,77] The experimental model which is most often used is that of the Mongolian gerbil. In this species, about half of the individuals lack arterial connections between cerebral and vertebral systems. After carotid occlusion and development of ischemia, HRP leaks

from the capillary lumen. In these investigations, there is no indication of endothelial cellular damage and, therefore, the extravasation of HRP is attributed to increased vesicular transport. The stimuli for this may be release of serotonin from platelets inducing vesicular formation secondary to vasoconstriction and increased blood pressure. Focal edema induced by a number of means, such as ultraviolet exposure, also increases vesicular transport and HRP uptake.

Nonionizing radiation has long been implicated in BBB disruption. Microwaves and x-rays have been studied extensively in this regard. The effects of microwaves are highly controversial. One report states that exposure of rats to 2450 MHz at 10 mW/cm^2 for two hours produces a marked extravasation.[96] This level is considered safe for human exposure in the United States, but not in other countries. The biological effects of microwaves can be subclassified as changes resulting from gross thermal effects or nonthermal effects. In this study, the body temperatures of the animals were constant and the altered permeability of the BBB was attributed to microwave-stimulated serotonin release from platelets, although other mechanisms are possible. Conversely, a recent publication indicates that much higher levels of radiation are required to cause protein leakage, specifically 3000 mW/cm^2.[97] At these levels, cerebral temperature increases significantly and changes can be attributed to gross thermal effects.

Porto-caval anastomosis, which causes severe liver dysfunction, has also been implicated in BBB breakdown.[54,77] This disintegration has been termed hepatic encephalopathy. As in a myriad of other circumstances, increased vesicular transport has been implicated as the mechanism of extravasation.

Several situations involving autoimmune afflictions and induced autoimmunity, such as experimental allergic encephalomyelitis (EAE) which is used as a model of multiple sclerosis, demonstrate an increased capillary permeability.[98] In EAE, the cerebral vessels are said to cuff or deform and this abnormality correlates with BBB breakdown. This may be important in the general progression of multiple sclerosis, as vascular changes are among the first changes which precede demylelination.

Agents which solubilize and fluidize membranes may also act to increase BBB permeability.[99,100,101] Dimethylsulfoxide (DMSO), in very high concentrations, has been thought to increase the flux of such plasma markers as inulin and mannitol. Its effects are said to be derived from its lytic action on membrane although interaction by micellular formation cannot be ruled out. Nortriptyline has a similar effect. Ethanol, in large doses, can also cause opening of the BBB to sucrose. It was postulated that this generalized increase in permeability may increase the susceptibility of the CNS to bacterial and viral infection. It has been shown, however, in acute and chronic doses of ethanol that were compatible with continued life, that there was no alteration in the BBB.[102,103]

Many heavy metals have been associated in both general and specific changes in the BBB.[1,56,104] Mercury (II) Hg^{+}, in high concentrations, i.e. >80 μm, causes a generalized increase in the permeability of cerebral capillaries to sugars and protein markers but in low concentrations affects only the hexose carrier. Ionic lead has a similar effect.

A number of conditions can alter BBB function in more subtle, yet no less damaging ways. In these instances, changes occur at the level of a specific carrier or carriers and, as such, only a particular type of compound is involved. These changes can result from physical alteration of

the carrier by denaturation. Additionally, diminution or increase of the blood level of a transportable compound can affect the utilization of the compound. In some cases, the rate limiting step in metabolism of a nutrient can be transferred from an enzyme to transit of the agent across the BBB.[21]

Glucose transport is very important to CNS function. Under normal circumstances, the rate-determining step in glucose metabolism involves the enzyme hexokinase. If, however, the plasma concentration of glucose falls, as in hypoglycemia, or cerebral metabolism increases, as in relative hypoglycemia, the limiting step in utilization is shifted to transport of glucose across the BBB.[20]

In severe hypoxia (pO_2 <10 mm), a number of progressive changes associated with glucose flux occur.[1] In the dog, after one minute of oxygen deprivation, the influx of glucose into the brain is unchanged but efflux decreases. Ten minutes after initiation of hypoxia, influx and efflux of glucose decrease. This has been explained by a postulated modulator which, in the absence of hypoxia, is bound to the carrier and alters transport. In other animal models, different results are obtained.

The transport of amino acids and, especially, neutral amino acids in certain disease states, has been the subject of much research. The neutral amino acid carrier is responsible for the transport of such neurotransmitter precursors as tryptophan, tyrosine and histidine and, as such, any interruption of this supply can have tremendous neurological consequences. Nowhere is this more apparent than in phenylketonuria.[1,20] This syndrome, which is associated with high blood levels of phenylalanine, has been linked to mental retardation. This condition of high phenylalanine levels can act to competitively inhibit other substrates of this carrier, such as tyrosine, tryptophan, and histidine. This is made possible because of

the similar Km values for these amino acids in addition to the similarity between the Km's and plasma levels of these amino acids. Hyperphenylalaninemia also can reduce protein synthesis by a similar mechanism. Treatment of this disease involves a phenylalanine restricted diet and 5-hydroxytryptophan supplements.

In hepatic encephalopathy, the neutral amino acid carrier is induced and, as previously discussed, this induction may be related to increase in ammonia and glutamine levels. Dihydroxyphenylalanine (L-DOPA), which is used as a dopamine source in the treatment of parkinsonism, is transported by the neutral amino acid carrier. It has been shown that ethanol increases DOPA transport as well as the transport of tyrosine, tryptophan and α-methyldopa into the CNS.[103]

The monocarboxylic acid carrier appears to be a major organ for eliminating metabolic acid wastes. As was previously discussed, the Km of lactate is close to its plasma concentration so that rises in lactate, such as those that accompany exercise, are only slowly translated to the CNS. In the case of anoxia, however, where cerebral levels of lactate rise, the systemic dissipation of this byproduct is slowed by the same phenomenon.[20] In the brain the loss of lactate is carrier and not diffusion-limited, unlike other organs. In hypoglycemia, lactate may act as an energy source.[105] This is true in neonates, where lactate uptake is much higher than in adults. The Km of the monocarboxylic acid carrier is also correspondingly higher in neonates.

Ketone bodies, which have an affinity for the monocarboxylic acid carrier, are produced during fasting and can act as a metabolic energy source. These bodies include β-hydroxybutyric acid and acetoacetic acid. The enzyme responsible for their production, β-hydroxybutyrate dehydrogenase, is

induced by starvation. The utilization of the surrogate food sources is limited by BBB transit.

In starvation, the monocarboxylic acid carrier is said to be induced, although this is controversial. A recent paper demonstrated a lower Km and V_{max} for carriers in starved animals compared with control animals.[33] The increased flux of β-hydroxybutyrate and other substrates was attributed in this article to an increase in the diffusional component.

A number of therapeutic agents have affinity for these carriers. These include such anionic compounds as probenicid, penicillin, and aspirin.[20] This affinity could potentially lead to competition and a decreased ability to eliminate metabolic acids from the CNS. It has been shown, however, that the concentrations required to cause inhibition are far above therapeutic levels.

The BBB is a complex system of enzymes, protein carriers, and vessels which impart to the brain a selective interface. This interface prevents potentially damaging substances from entering the brain without impeding the entry of nutrients or the exit or metabolites and excretory products. In adverse circumstances, the permeability of the barrier can be increased, resulting in a number of deleterious effects.

Prodrugs and Drug Delivery Systems

The BBB excludes a number of pharmacologically active agents and, as such, treatment of many cerebral diseases is severely limited. In order to increase the effectiveness of drugs which are active against central maladies, the pharmacokinetic profile of the agent must be augmented and, specifically, the transit time of the drug in the brain must be increased. If a method were available to implement these alterations, an agent could be delivered specifically to the brain. This specificity should increase

the therapeutic index of an agent since not only is the concentration of the agent increased in the vicinity of the bioreceptor but, of equal importance, the peripheral concentration of the drug is reduced decreasing any associated toxicity.

Unfortunately, there are very few methods for circumventing the BBB and these are of limited usefulness. The direct administration of drugs into the CNS;i.e., an intrathecal injection has been used to deliver the folate antagonist, methotrexate, to the brain. This method is not very satisfactory since the distribution of methotrexate in the brain is uneven and slow.[46] Additionally, since the ventricular volume of the CSF is small, increases in intracerebral pressure can occur with repeated injections. This is particularly dangerous when the intracerebral pressure is already high as it is in CNS cancers. Repeated lumbar puncturing also carries a risk.

A general method which can be applied to delivery of drugs to the brain is the prodrug approach.[106-110] The term prodrug was coined by Albert and refers to the result of a transient chemical modification of a pharmacologically active agent. This change imparts to the compound an improvement in some deficient physiochemical property such as water solubility or membrane permeability. Ideally, a prodrug is biologically inactive but reverts to the parent compound in vivo. This transformation can be mediated by an enzyme or may occur chemically due to some designed instability in the agent. The aim of these manipulations is to increase the concentration of the active agent at its site of action and thereby, increase its efficacy. While potentially there are many different types of proderivatives, most thus far synthesized are simple esters and amides. These compounds are transformed to the parent acid, alcohol or amine by the ubiquitous hydrolases which are present in vivo. Many anticancer

agents have lent themselves to this type of manipulation. Several amides, for example, of the highly water soluble anticancer agent guanazole have been made. This series of lipoidal compounds hydrolyzed in vivo to yield the parent drug.[111] A more sophisticated prodrug is cyclophosphamide, which is inactive in vitro. The agent is activated by P450 mixed function oxidases in the liver to the potent alkylating agent, N,N-bis(chloroethyl)-phosphordiamidic acid.[109] This drug is extensively used in cancer chemotherapy.

One of the most important applications of prodrugs is in the sustained release of therapeutically active agents. Cytosine arabinoside is used as an S-phase specific antimetabolite, but suffers from rapid metabolism by cytidine deaminase requiring a continual administration of the drug. This problem produced the prodrug cyclocytidine which is not a substrate for cytidine deaminase and which slowly releases the cytosine arabinoside by ring cleavage.[112]

The influx of a compound to any organ can be related by the equation

$$K_p = QE$$

where K_p is the clearance of a drug by an organ, Q is the blood flow through that organ and E is the extraction coefficient.[113] The extraction coefficient is related to the lipophilicity or octanol-water partition coefficient of a compound which is in turn related to the ability of a compound to partition into phospholipoidal membranes and consequently into organs. By increasing the lipophilicity of a compound with the prodrug approach, one can increase the entry of a compound into its site of action but this is not specific and, in general, all organs are exposed to a greater tissue burden.[113] Nonspecificity is, therefore, one of the major drawbacks of the prodrug approach. This is especially important

with cytotoxic agents. While increased membrane permeability makes a compound more effective locally as a cytotoxic agent, there is almost always a disproportionate rise in systemic toxicity. This has severely restricted anticancer prodrugs of this type.

Several types of toxicities are also associated with prodrugs.[114] Theoretically, a prodrug should be metabolized only to the parent compound but the formation of toxic metabolites by the prodrug is possible. This occurs with such compounds as phenacetin, a prodrug of acetaminophen. Another possible toxic reaction may be brought about by enzymatic or glutathione depletion. The compound thiamine tetrahydrofurfuryl disulfide, a prodrug of thiamine, requires glutathione-mediated disulfide bond cleavage for activation and, therefore, toxicity may arise from the associated depletion of glutathione.

The idea of using prodrugs to increase the specificity of delivery has been considered. This has proved, however, to be difficult and not very fruitful. Two basic approaches have been taken in this regard: site-directed or site-activated delivery and delivery by the association of a drug with a macromolecular carrier.

The first method does not attempt to concentrate a compound at a particular location, but is based on site-specific activation of the prodrug. For this to be possible, the enzyme responsible for the activation must be located specifically, or at least in high relative concentrations in a particular organ. The finding, for example, that γ-glutamyl peptidase is present in high concentrations in the kidney led to a number of compounds substituted with the γ-glutamyl group.[113] Sulfamethiazide and L-DOPA were derivatized in this manner in order to achieve renal delivery.

This approach has been extensively applied to cancer chemotherapy.[114] The philosophy behind this application is related to the many differences that occur between cancer cells and normal cells. These variations are basically the result of the altered metabolism of neoplastic cells. Many alkylating agents have been synthesized in an attempt to capitalize on these differences. The lower pH of tumor cells has been exploited in a series of aziridines which are more active at the pH of tumors than at physiological pH. The greater reducing power of cancerous sites has led to the development of a number of biologically inactive azo compounds which upon reduction yield potent cytotoxic agents.[115] Examples of these are tetrazolium mustard and azomustard, both of which are reduced to aniline mustards. The inactivity of the parent compound is due to the delocalization of the nucleophilic nitrogen lone pair by the conjugated ring system.

A number of O-phosphate esters have been synthesized in order to take advantage of the high levels of acid phosphatase which are characteristic of human neoplasms.[115] The O-phosphate esters of p-hydroxy mustard and estradiol mustard were prepared as specific agents to be used in prostate cancers. The enzyme, γ-glutamyl transpeptidase, is also found in high concentrations in tumor cells so that γ-glutamyl derivatives of cytosine arabinoside and phenylene-diamine mustard have been proposed. The presence of hydrolytic esters has also been established in neoplastic formations. These include esterases and β-glucuronidases.[112,115] The cytotoxic agent, aniline mustard, is converted in the liver as a result of a first pass effect to its O-glucuronide. Tumors which contain high β-glucuronidase activity convert the O-glucuronide to the potent alkylating agent p-hydroxyaniline mustard.

While these compounds have demonstrated some promise as anticancer drugs, for the most part they have not lived up to their potential. There are a number of reasons for this failing. The chemical manipulation of these agents may act to decrease their accessibility to a site of action. Additionally, if the agent is fairly lipophilic, it may "leak" from its site of action before exerting a pharmacological effect.[113] In cancers there may also be diffusion limitations because of the restrictions in blood flow.

A second approach for increasing the specificity of an agent for a particular organ involves the coupling of a pharmacologically active agent with a macromolecule.[116] The specificity derived from such a system is related to the interaction between cells and endogenous and exogenous biopolymers and macromolecules. Albumin, for example, is actively endocytosed by various macrophages.[117] An anticancer compound could be coupled to albumin and the complex taken up by a macrophage tumor. This would then be directed to a lysosome where the drug would be hydrolyzed from its albumin carrier and exert its pharmacological action specifically. This has also been suggested as a means of treating DNA viruses which implant in macrophages. Anthracyclines, such as daunorubicin and adriamycin, intercalate into DNA. A drug carrier has been devised in which fragments of DNA containing intercalated anthracyclines are administered systemically.[114,118] This intercalated complex is inactive but can be endocytosed and broken down in lysosomes by DNAases. Again, the aim is to release the cytotoxic agent in the vicinity of the malignancy and thereby increase its efficacy. Antibodies have also been studied as specific delivery carriers, but research has been hampered by the inhomogeneity of tumor-specific antibodies.

The idea of including a drug into liposomes formed in vitro has received a great deal of attention.[117,119] In these systems, the drug is inactive since it is enclosed in the phospholipoidal matrix of the liposome and, as in the case of the albumin conjugates, these packets are taken up by endocytosis. Again, the system should specifically exert its action at the site of influx. While these approaches are promising theoretically, they have not met with much success. These carrier complexes are the subject of several recent books.[117,119]

A general method was recently proposed for the specific delivery of drugs to the brain. This system was based on the results of some work with N-methylpyridinium-2-carbaldoxime chloride (2-PAM).[120,121,122,123] This pyridinium quaternary compound is the agent of choice for the treatment of organophosphate poisonings, and exerts its action by reactivating deactivated cholinesterases. The problem with this agent is its highly polar nature. Organophosphates such as diisopropyl fluorophosphate (DFP) and paraoxon are very lipophilic and easily penetrate the BBB. The brain is, therefore, very susceptible to acetylcholinesterase inactivation by these agents. The highly polar 2-PAM has a very low activity in the brain since it is almost totally excluded by the BBB. To deal with this problem the dihydro adduct of 2-PAM, pro-2-PAM, was synthesized as a prodrug.

2-PAM pro-2-PAM

The rather involved synthesis of pro-2-PAM yielded the enamine salt which corresponds to the 3-protonated dihydro compound. The pK_a of the tertiary nitrogen in this enamine salt was determined potentiometrically and spectrophotometrically to be 6.32 ± 0.6 and the oxime was estimated to have a pK_a of ~11. With a pK_a of 6.32, it would be predicted that about 90% of the enamine would exist as the free base at physiological pH (7.4). Since this dihydro free base is far more lipophilic than the parent quaternary, its ability to penetrate membranes is greatly enhanced. This pro-2-PAM is rapidly converted to the parent 2-PAM at physiological pH and the $t_{\frac{1}{2}}$ has been determined to be 1.04 min by pharmacokinetic modeling. This rapid conversion of the pro-2-PAM to 2-PAM is desirable since ideally, the only metabolism of the prodrug is to the parent compound.

A number of experiments were carried out using 2-PAM and pro-2-PAM. From the linear descending portion of a semilog plot of blood concentration versus time, the biological $t_{\frac{1}{2}}$ of 2-PAM and pro-2-PAM was calculated. The $t_{\frac{1}{2}}$ for comparable doses of 2-PAM and pro-2-PAM differed by more than 60 min, and since the conversion of pro-2-PAM → 2-PAM is rapid, this difference was assumed to be the altered distribution of pro-2-PAM. This indicates that even though the rate of conversion of pro-2-PAM is rapid, it is long enough for the enhanced distributional characteristics of the pro-2-PAM to be expressed.

Blood levels of 2-PAM were significantly higher after an oral dose of pro-2-PAM than with a dose of 2-PAM. If pro-2-PAM is administered intravenously, there are no new metabolites formed. If brain concentrations of 2-PAM are examined after 2-PAM and pro-2-PAM dosing, the concentration of 2-PAM in the brain is 13-fold higher in the case of pro-2-PAM administration. If brain acetylcholinesterase is inactivated using DFP, the

extent of reactivation observed in mice injected with pro-2-PAM shows a dramatic increase over 2-PAM treated animals.

A further experiment with 2-PAM showed that this small quaternary salt was rapidly lost from the CNS and this loss was attributed to an active efflux process.[124] These results differ from those obtained by Ross and Froden who attempted to deliver a quaternary compound to the brain as its uncyclized ω-haloalkyl amine.[125,126] They found that the loss of the quaternary compound was slow ($t_{\frac{1}{2}}$ = 38 hours) and concluded that the efflux of the quaternary was comparable to its influx. This difference in the rate efflux may be related to differences in molecular size, shape or charge.

This preliminary work led to a proposed drug delivery system (Figure 1-2) which is specific for the brain and which is generally applicable.[124] In this proposal, a pharmacologically active agent, whose ability to pass the BBB is low, is chemically linked to a pyridinium carrier. This carrier could be envisioned as a nicotinamide or nicotinic acid ester. This complex would be reduced under conditions which would yield the dihydropyridine. This complex is then injected systemically and, because of the increased lipophilicity of the dihydropyridine, partitions into the brain as well as into the periphery. In both locations, oxidation (k_{ox}) should occur. The rate of this oxidation is somewhat controllable by the judicious placement of ring substituents on the pyridinium nucleus. Systemically, the charged polar oxidized species should be eliminated rapidly by the kidney and/or liver (k_{out2}), while in the brain the compound, because of its charge and size, would be retained i.e., $k_{out2} > k_{out1}$.

Also, in both locations, cleavage of the drug from its carrier should occur ($k_{cleavage}$). In the brain, the small nontoxic pyridinium carrier is

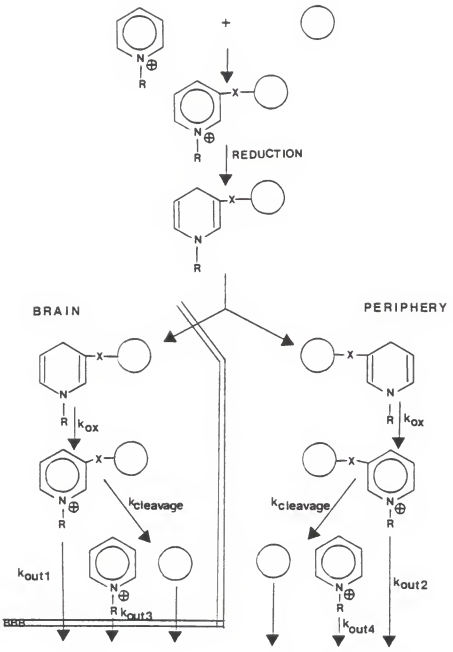


Figure 1-2. A Proposed Carrier-mediated Chemical Delivery System with Specificity for the Brain. The Drug Molecule to be Transported is Represented by the (O).

rapidly eliminated, k_{out3}. If the cleavage of the drug from its carrier occurs at an appropriate rate i.e., $k_{cleavage} > k_{out1}$ a sustained release of the agent to the brain could also be obtained. Again, the cleaved polar drug would be rapidly eliminated systemically. By these manipulations a compound can be delivered specifically to the brain while its systemic concentration is kept low. This reduces any associated systemic toxicity of the agent and increases its therapeutic index dramatically.

In this system the positively charged carrier complex cleaves to yield the active compound. The dihydropyridine is not, therefore, a prodrug but rather a pro-prodrug or, better stated, a chemical delivery system. This drug delivery system is based on the naturally occurring reduced nicotinamide adenine dinucleotide (NADH) $\rightleftarrows$ oxidized nicotinamide (NAD^+) system. These endogenous coenzymes are important in electron transferring chains and their suitability to this purpose is related to the chemistry of dihydropyridines and enamines. In the described delivery system the relative unstability, greater lipophilicity and predictability of chemistry of the dihydromoieties are exploited. Additionally, since this method relies on enzymatic activation by an endogenous system (NADH dehydrogenase) whose substrates closely approximate the delivery compounds, any toxicity associated with the oxidation should be minimal. In this system the BBB has not been simply circumvented but rather used as an integral part of the delivery scheme.

Statement of the Problem

The chemical delivery system proposed by Bodor et al. should demonstrate a broad applicability since the agent to be delivered is simply attached to a particular carrier.[124] In certain instances, however, this system can be simplified. If a pyridinium nucleus is an integral structural

component of a molecule, as it is in many pharmacologically active agents, the molecule is provided with an internal delivery moiety. The drug to be delivered and the carrier are therefore merged into one molecule. The pharmacokinetics of this scheme is also simpler than those involved with the carrier system since no cleavage is necessary of the delivered molecule from the carrier. In this approach, which appears in Figure 1-3, an appropriately chosen pharmacologically active compound i.e., one which contains a pyridinium moiety, would be reduced to its corresponding dihydropyridine. This lipophilic species would penetrate the BBB as well as into the systemic circulation. After a period of time determined by the stability of the compound, it would be oxidized to the parent quaternary salt (k_{ox}). Systemically, this agent would rapidly be eliminated by filtration or by tubular secretory mechanisms (k_{out2}). In the CNS, however, since the ability of the compound to freely diffuse would be lost, it would be delivered fairly specifically ($k_{out2} > k_{out1}$). The transit time of the drug in the brain would depend upon a number of factors including the participation of the compound in any active efflux processes. It would be hoped that in most cases the rate at which the compound entered the brain would be faster and ideally, much faster than the rate at which the compound left the brain (k_{out1}).

This system, unlike the carrier mediated chemical delivery system, can be considered a prodrug. This prodrug should, however, demonstrate a specificity for the brain because of its design, the characteristics of the BBB, and the chemistry of dihydropyridines. Again, this specificity should increase the therapeutic index of the drug delivered.

Several criteria were used in choosing a molecule for these delivery approaches. Obviously, the compound should contain a pyridinium moiety

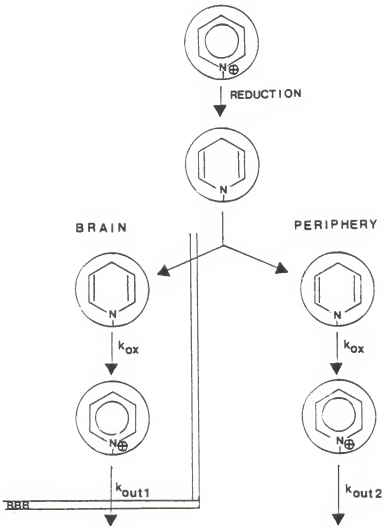
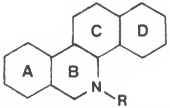
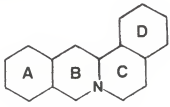


Figure 1-3. The Proposed Drug Delivery System.

which is reducible to some dihydropyridine species which is stable enough to be isolated. The compound should be active in vitro. Since quaternary compounds are to be considered, the lack of any in vivo activity might be ascribed to transport or distributional problems. A review of the literature showed two groups of compounds which looked particularly suitable. These include the substituted benzophenanthridinium salts and the protoberbine alkaloids which have the basic skeleton:

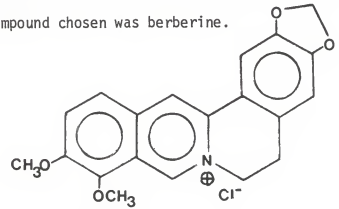


benzophenanthridinium ion



protoberbine

Members of these groups contain an N-substituted isoquinoline moiety which can be reduced by a number of agents. A number of these dihydroisoquinoline compounds are stable.[127] These agents show a wide range of effects including antineoplastic and antibiotic activity.[128-136] The specific compound chosen was berberine.



berberine

Berberine, which has the chemical name 5,6-dihydro-9,10-dimethoxybenz-[g]-[1,3]benzodioxolo[5,6-a]quinolizinium chloride, has a rather high in vitro activity against several cancer types including Ehrlich and lymphoma ascites.[131,133] Its in vivo action is, however, very low.[132,133]

Berberine is widely distributed in the plant kingdom and is found in such families as menispermaceae and berberidaceae, to name only two.[137] The compound was isolated by 1826 by Pelletan and Chevallier.

Biosynthetically, berberine is interesting because of the presence of the so-called berberine bridge. This term refers to the single carbon atom between the nitrogen and the methoxylated aromatic nucleus. Two postulates for the formation of this have been suggested. The first involves formaldehyde in a Mannich-type ring closure while the second involves an oxidative cyclization of an N-methyl group.[138,139] Labelling experiments favor the second mechanism.[139]

There are several total syntheses for berberine in the literature and the chemistry of the protoberbine alkaloids is well reviewed.[140-142] Berberine has a variety of pharmacological actions and several therapeutic uses. Pharmacologically, it exhibits a depressive action on excitable tissues,[143] induces hypotension and tachycardia,[144,145] and inhibits a number of enzymes including histaminease (human pregnancy plasma diamine oxidase),[146] cholinesterase,[144] dopamine-adenyl cyclase,[147] and cation-dependent ATP phosphorylases.[148] Berberine also possesses an antiheparin[149] and local anesthetic activity.[144] Because of the affinity of berberine for dopaminergic receptors[150] and alcohol dehydrogenases,[151] it has been used to characterize geometric and stereospecific requirements for substrate binding to these enzymes. Berberine has long been known as an antibiotic. The alkaloid causes mutations in certain bacterium by affecting nonchromosomal genetic material.[134] Berberine has been used mostly in India to treat cholera,[152] diarrhea,[153] leshmaniasis and other parasitic infections.

Biochemically, berberine acts to inhibit DNA, RNA and protein synthesis.[143] It has been suggested that many compounds which are structurally similar to berberine exert their cytotoxic activity via alkylation at the iminium site.[128] A more thoroughly studied, and perhaps more accurate, hypothesis is that berberine acts by intercalating between the base pairs of DNA. This intercalation can be explained by a modified neighbor-exclusion model.[154] A model which has been used to explain the intercalation of berberine is one in which the greater portion of rings A, B and D are intercalated.[155] As berberine complexes, it assumes a more rigid planar species as may be indicated by the increase in fluorescence quantum yield. This intercalation does, however, slightly bend the double helix. The unwinding angle of DNA due to intercalation of berberine is less than for the more planar molecules such as corylene. The effect of the positive charge on intercalation has also been noted.[156]

Berberine, therefore, is appropriately suited as a candidate for inclusion in the drug delivery system described. In order to verify the original hypothesis of site-specific delivery, a number of experiments are required. Dihydroberberine must be synthesized and its stability assayed. The ability of dihydroberberine to penetrate the BBB and concentrate in the brain must also be demonstrated. Additionally, the toxicity and anticancer activity of the agent should be investigated.

CHAPTER 2

MATERIALS AND METHODS

Elemental analyses of compounds synthesized were performed by Galbraith Laboratories, Inc., Knoxville, Tennesse, or Atlantic Microlab, Inc., Atlanta, Georgia. Uncorrected melting points were determined using a Thomas-Hoover melting point apparatus. Ultraviolet spectra (UV) were recorded on a Beckman 25, Cary 210 or Cary 219 spectrophotometer. An Apple II Plus microprocessor was dedicated to the Cary 210 instrument. Infrared spectra (IR) were taken on a Beckman 4210 high resolution and a Beckman Acculab 1 infrared spectrophotometer. Samples were analyzed as a thin film between sodium chloride windows or as a potassium bromide pellet. Nuclear magnetic resonance spectra (NMR) were obtained from either a Varian T60 or Joel-JNM-FX 100 Fourier transform spectrometer. The samples were dissolved in deuterated chloroform ($CDCl_3$), deuterated dimethylsulfoxide ($(CD_3)_2SO$), deuterated pyridine (C_5D_5N), deuterated methanol (CD_3OD), deuterated acetonitrile (CD_3CN), deuterium oxide (D_2O) or trifluoroacetic acid (TFA). Chemical shifts in parts per million were reported relative to the internal standard tetramethylsilane except in aqueous systems where sodium 3-trimethylsilylpropanesulfonate is used. Mass spectra were obtained using a DuPont 21-491B double focusing magnetic sector mass spectrometer to which was dedicated a Hewlett Packard 2100A computer. In all determinations, the ionizing voltage was 70 eV.

High pressure liquid chromatography (HPLC) was performed on either a Waters Associates system consisting of a Model U6K injector, a Model 6000A solvent delivery system and a Model 440 absorbance detector or a

ternary Beckman system consisting of three Model 112 solvent delivery systems, a Model 160 absorbance detector and a Model 421 controller. In some studies, a Varian Fluorichrom^{R} detector was used with one of the Beckman pumps. Thin-layer chromatography (TLC) was performed on EM Reagents Cat. 5751 Aluminum Oxide 60 F-254 precoated plates (layer thickness 0.25 mm) or Analtech, Inc. Uniplate^{R}, which are precoated with silica gel G at a thickness of 0.25 mm. Tissues were homogenized by a VirTis 45 homogenizer or by a teflon pestle and ground glass tube. In potentiometric titrations, a Radiometer-Copenhagen PHM 84 Research pH meter was used. A Sage Instrument Co. Model 341A syringe pump was used for infusions. For radionuclide studies, a Packard Tri-Carb 460 CD Liquid Scintillation system was employed.

In the theoretical studies, the MINDO/3 program was modified to suit the University of Florida's IBM System/370 computer. The molecule drawing program, X3DMOL, was developed by E.W. Phillips.

All chemicals used were of reagent grade. Berberine was obtained from Sigma Co. while nicotinic acid, nicotinamide, methyl iodide, benzyl bromide, butanol, hexanol, octanol, decanol, sodium borohydride and sodium dithionite were obtained from Aldrich Co. Phenethyl alcohol was purchased from Matheson, Coleman and Bell. Tritiated inulin, Soluene^{R} and scintillation cocktails were obtained from New England Nuclear. Solvents were of chromatographic purity and were obtained from Fisher Co. Pyridine (Aldrich Co.) was refluxed over CaH_2 and distilled before using. In cases where oxygen was to be excluded, solutions were made from water which, after boiling for fifteen minutes, was cooled with a stream of pyrogallol-scrubbed nitrogen passing through it. A phosphate buffer (pH 7) was made by dissolving 1.183 g of potassium dihydrogen phosphate and 4.320 g of disodium hydrogen phosphate in water and diluting to 1.0 ℓ.

Synthesis

Dihydroberberine (2)

Seven grams of berberine chloride (1) were dried over phosphorous pentoxide at 80°C in a vacuum oven for eight hours. Five grams of the dried salt (0.013 moles) were added to a suspension of dry pyridine and 0.6 g of sodium borohydride ($NaBH_4$). The solution was stirred under N_2 for twenty minutes at room temperature, at which time 0.5 g more of $NaBH_4$ were added. The liquid was then poured into 800 ml of ice water. The ensuing precipitate was dried overnight at 50°C over phosphorous pentoxide. The crude material was recrystallized from benzene-petroleum ether (low boiling). The yield was 35%: Melting point 156-158°C, Literature 157-159°C; ^1H NMR ($CDCl_3$) δ 7.1 (1 H, s), 6.7 (2 H, s), 6.4 (1 H, s), 5.9 (3 H, s), 4.3 (2 H, s), 3.4 (6 H, s), 3.1 (2 H, t), 2.9 (2 H, t); MS m/e 338 (M^+ + 1) 22%, 337 (M^+) 100%, 322 (M^+ - 15) 27%, 278 (M^+ - 59) 13%; UV (95% ethanol) 370 λ_{max}; IR (KBr) ν (C-H) 3005 and 2970, (C-C, C-N) 1600 and 1482, (C-O-C asym) 1227 and 1270, (C-O-C sym) 1028 and 1083; ^{13}C NMR (see figure 3-6); Elemental analysis calculated %: C, 71.21; H, 5.60; N, 4.15. Found %: C, 71.19; H, 5.70; N, 4.14.

Dihydroberberine Hydrochloride (3)

One gram of (2) was dissolved in a minimal amount of CH_2Cl_2. Anhydrous hydrogen chloride, produced by dropping concentrated sulfuric acid (H_2SO_4) on sodium chloride (NaCl), was bubbled through the solution yielding a yellow precipitate. The material was recrystallized in aqueous ethanol.

9-Demethylberberine (Berberrubin) (4)

Seven grams of (1) were dried for two hours at 100°C in a vacuum oven and then were heated to 200°C for an additional thirty minutes. The deep purple material produced was dissolved in hot water and extracted with

chloroform ($CHCl_3$). The organic layer was reduced to dryness and the residue dissolved in hot water. The solution was filtered and then made acidic with an excess of hydrochloric acid (HCl). The ensuing precipitate (yellow-brown solid) was collected by filtration and redissolved in hot water. The solution was filtered and made basic with potassium hydroxide. The solution turned deep purple and crystallization was induced by scratching: ^1H NMR (CD_3OD) δ 9.0 (s, 1 H), 7.8 (s, 1 H), 7.2 (m, 2 H), 6.8 (m, 2 H), 5.8 (s, 2 H), 3.7 (s, 3 H), 4.4 (m, 2 H), 3.0 (m, 2 H); UV (95% ethanol) 276 nm and 238 nm λ_{max}, others 512 nm; IR (KBr) ν (C-H) 3000 and 2900, (C-C, C-N) 1635, 1571, 1509 and 1472, (C-O-C asym) 1221 and 1289, (C-O-C sym) 1035 and 1144; Elemental analysis calculated %: C, 71.03; H, 4.67; N, 4.36. Found %: C, 71.20; H, 4.81; N, 4.42.

Berberine Iodide (5)

A solution of 1.0 g of (4) in acetone was prepared. A 1.0 M excess of methyl iodide was added and the solution was allowed to reflux for several hours. The characteristic yellow color of berberine appeared and TLC, NMR, IR, and UV confirmed the methylation.

3-(Aminocarbonyl)-1-methylpyridinium Iodide/1-Methylnicotinamide Iodide (6)*

Five grams of nicotinamide (0.041 moles) were dissolved in 50 ml of dry methanol. A molar excess of methyl iodide (11.6 g) was added to the stirring mixture and after one hour of refluxing, a precipitate formed. This was filtered and washed. The material was recrystallized from aqueous methanol: Melting point 101-103°C, Literature 102-105°C; UV (H_2O) 224 mm and 265 mm λ_{max}; NMR and IR were identical with the literature.

*The nicotinic acid derivatives are given a systematic name followed by a common name

3-(Aminocarbonyl)-1-(phenylmethyl)pyridinium Bromide/1-Benzylnicotinamide Bromide (7)

Ten grams of nicotinamide (0.083 moles) were dissolved in 150 ml of methanol. A molar excess of benzyl bromide (28.0 g) was added and the mixture was allowed to reflux for several hours. Upon cooling, a white solid appeared. This was filtered, washed and recrystallized from methanol: Melting point 206-208°C, Literature 205°C; ^1H NMR (D_2O or TFA) δ 9.5 (1 H, s), 9.0 (2 H, m), 8.2 (1 H, m), 7.5 (5 H, s), 5.9 (2 H, s); IR (NaCl) ν (N-H asym) 3300, (N-H sym) 3148, (-C(=O)NH_2 Amide I) 1690, (Amide II) 1643, (C-N-C) 1388.

3-Pyridinecarboxylic Acid Ethyl Ester/Ethyl Nicotinate (8)

Forty-two grams (0.34 moles) of nicotinic acid were mixed with 55 ml of absolute ethanol and 25 ml of concentrated H_2SO_4. The mixture was refluxed in an oil bath for four hours at which time the solution was poured over ice and made slightly basic with ammonia. The aqueous solution was extracted with ethyl ether. The organic layer was dried with sodium sulfate (Na_2SO_4) and the solvent evaporated under reduced pressure. The product was a clear liquid and the yield was 55%: ^1H NMR ($CDCl_3$) δ 9.2 (1 H, s), 8.8 (1 H, m), 8.2 (1 H, m), 7.4 (1 H, m), 4.4 (2 H, q), 1.4 (3 H, t); IR (NaCl) ν (C-H) 2990, (C=O) 1728, (-C-C(=O)O) 1286, (O-C-C) 1112, (γ CH) 742, (β ring) 703.

3-Pyridinecarboxylic Acid Butyl Ester/Butyl Nicotinate (9)

Forty-two grams (0.34 moles) of nicotinic acid were dissolved in 55 ml of 1-butanol and 25 ml of concentrated H_2SO_4 was slowly added. The solution was heated to reflux in an oil bath for three hours, at which time the solution was poured over ice and made slightly basic with ammonia. The mixture was extracted with ethyl ether. The separated ether layer was dried over Na_2SO_4 and the solvent removed under reduced pressure.

The product was a clear liquid and the yield was 62%: ^{1}H NMR ($CDCl_3$) 9.2 (1 H, s), 8.8 (1 H, m), 8.2 (1 H, m), 7.4 (1 H, m), 4.3 (2 H, t), 1.6 (4 H, m), 0.97 (3 H, t); IR (NaCl) ν (C-H) 2962, (C=O) 1730, (C-C, C-N) 1594, (-C-C(=O)O) 1288, (O-C-C) 1117, (γ CH) 742, (β ring) 702.

3-Pyridinecarbonylchloride Hydrochloride/Nicotinoyl Chloride Hydrochloride (10)

Forty-one grams (0.33 moles) of nicotinic acid were stirred in an ice bath with 110 ml of thionyl chloride ($SOCl_2$) slowly added. After the addition was complete, the mixture was refluxed for three hours. The $SOCl_2$ was removed under reduced pressure and traces of $SOCl_2$ were azeotroped off with benzene. The white crystalline product was obtained in 94% yield; NMR and IR were identical with the literature.

3-Pyridinecarboxylic Acid Hexyl Ester/Hexyl Nicotinate (11)

Ten grams of (10) (0.062 moles) were dissolved in 100 ml of dry distilled pyridine and 5.73 ml (0.062 moles) of 1-hexanol. The solution was refluxed in an oil bath for six hours. The solution was then poured over ice which had been made basic with ammonia. The aqueous solution was extracted with ethyl ether, the organic layer dried over Na_2SO_4, and the solvent removed under reduced pressure. The yield was 60%: ^{1}H NMR ($CDCl_3$) δ 9.1 (1 H, s), 8.7 (1 H, m), 8.2 (1 H, m), 7.3 (1 H, m), 4.3 (2 H, t), 1.2 (8 H, m), 0.87 (3 H, t); IR (NaCl) ν (C-H) 2938 and 2961, (C=O) 1726, (C-C, C-N) 1590, (-C-C(=O)O) 1282, (O-C-C) 1111, (γ CH) 741, (β ring) 702.

3-Pyridinecarboxylic Acid Octyl Ester/Octyl Nicotinate (12)

Ten grams of (10) (0.062 moles) were dissolved in 100 ml of dry pyridine and 9.75 ml (0.062 moles) of 1-octanol. The solution was heated to reflux in an oil bath and the progress of the reaction monitored with TLC. After eight hours, the liquid was poured over ice which had been made basic with ammonia. The aqueous solution was extracted with ethyl ether. The

ether layer was dried over sodium sulfate (Na_2SO_4) and the solvent removed under reduced pressure. The yield was 58%: ^1H NMR ($CDCl_3$) δ 9.1 (1 H, s), 8.7 (1 H, m), 8.2 (1 H, m), 7.3 (1 H, m), 4.3 (2 H, t), 1.3 (12 H, m), 0.88 (3 H, t); IR (NaCl) ν (C-H) 2920 and 2950, (C=O) 1723, (C-C, C-N) 1589, (-C-C(=O)O) 1277, (O-C-C) 1109, (γ CH) 732, (β ring) 693.

3-Pyridinecarboxylic Acid Decyl Ester/Decyl Nicotinate (13)

Ten grams of (10) (0.062 moles) were dissolved in 100 ml of dry pyridine and 11.82 ml (0.062 moles) of 1-decanol. The solution was refluxed in an oil bath for eight hours. The liquid was then poured over ice which had been made basic with ammonia. The aqueous solution was extracted with ethyl ether, the organic layer dried over Na_2SO_4, and the solvent removed under reduced pressure. The yield was 64%: ^1H NMR ($CDCl_3$) δ 9.2 (1 H, s), 8.7 (1 H, m), 8.2 (1 H, m), 7.2 (1 H, m), 4.3 (2 H, t), 1.2 (16 H, m), 0.83 (3 H, t); IR (NaCl) ν (C-H) 2915 and 2946, (C=O) 1720, (C-C, C-N) 1584, (-C-C(=O)O) 1275, (O-C-C) 1105, (γ CH) 732, (β ring) 692.

3-Pyridinecarboxylic Acid β-Phenylethyl Ester/β-Phenethyl Nicotinate (14)

Twenty-six grams (0.15 moles) of (10) were dissolved in 200 ml of pyridine. To this stirring solution was added dropwise 18.3 g (0.15 moles) of β-phenethyl alcohol. The solution was refluxed in an oil bath for several hours. The solution was then poured over ice and made slightly basic with ammonia. This solution was then extracted with ether. The organic layer was dried over Na_2SO_4 and evaporated under reduced pressure. The yield was 62%: ^1H NMR ($CDCl_3$) δ 9.1 (1 H, s), 8.6 (1 H, m), 8.0 (1 H, m), 7.2 (6 H, s), 4.4 (2 H, t), 2.9 (2 H, t); IR (NaCl) ν (C-H) 3030 and 2959, (C=O) 1722, (C-C, C-N) 1585, (-C-C(=O)O) 1276, (O-C-C) d 1120, 1105, (γ CH) 737, (β ring) 695.

3-(Ethoxycarbonyl)-1-methylpyridinium Iodide/Ethyl N-Methylnicotinate Iodide (15)

Ten grams of (8) (0.066 moles) were mixed with methanol and with a molar excess of methyl iodide (18.7 g). The solution was refluxed for several hours. The solvent was removed, yielding a red-orange oil which solidified on cooling to a yellow solid. The solid was recrystallized from acetone-ether: ^{1}H NMR ($CDCl_3$, $(CD_3)_2SO$), δ 9.6 (2 H, m), 9.0 (1 H, m), 8.4 (1 H, m), 4.8 (3 H, s), 4.5 (2 H, q), 1.4 (3 H, t); IR (NaCl) ν (C-H) 3008, (C=O) 1723, (-C-C(=O)O) 1303, (O-C-C) d 1102, 1117, (γ CH) 742, (β ring) 654.

3-(Butoxycarbonyl)-1-methylpyridinium Iodide/Butyl N-Methylnicotinate Iodide (16)

Ten grams of (9) (0.056 moles) were dissolved in 60 ml of acetone and a molar excess of methyl iodide (15.9 g) was added. The solution was refluxed for two hours. The solvent was removed, leaving a yellow solid which was recrystallized from acetone-ether: ^{1}H NMR ($CDCl_3$, $(CD_3)_2SO$) δ 9.9 (2 H, m), 9.0 (1 H, m), 8.6 (1 H, m), 4.5 (3 H, s), 4.4 (2 H, t), 0.93 (3 H, t); IR (NaCl) ν (C-H) 2950, (C=O) 1720, (-C-C(=O)O) 1291, (O-C-C) 1100, (γ CH) 735, (β ring) 651.

3-(Hexoxycarbonyl)-1-methylpyridinium Iodide/Hexyl N-Methylnicotinate Iodide (17)

Ten grams of (11) (0.048 moles) were dissolved in 60 ml of acetone, and a molar excess of methyl iodide (13.7 g) was added. The solution was refluxed for two hours. The solvent was removed, producing an orange oil: ^{1}H NMR ($CDCl_3$, $(CD_3)_2SO$) δ 9.3 (2 H, m), 8.9 (1 H, m), 8.3 (1 H, m), 4.7 (3 H, s), 4.3 (2 H, t), 0.90 (3 H, t); IR (NaCl) ν (C-H) 2944, (C=O) 1723, (-C-C(=O)O) 1295, (O-C-C) 1111, (γ CH) 738, (β ring) 654.

3-(Octoxycarbonyl)-1-methylpyridinium Iodide/Octyl N-Methylnicotinate Iodide (18)

Ten grams of (12) (0.043 moles) were mixed with 60 ml of acetone and with a molar excess of methyl iodide (12.1 g). The solution was allowed to reflux for two hours at which time the solvent was removed, yielding an oil which was resistant to crystallization: ^{1}H NMR ($CDCl_3$, $(CD_3)_2SO$) δ 9.9 (2 H, m), 9.0 (1 H, m), 8.6 (1 H, m), 4.6 (3 H, s), 4.4 (2 H, t), 0.92 (3 H, t); IR (NaCl) ν (C-H) 2960, (C=O) 1731, (-C-C(=O)O) 1302, (O-C-C) 1120, (γ CH) 749, (β ring) 668.

3-(Decoxycarbonyl)-1-methylpyridinium Iodide/Decyl N-Methylnicotinate Iodide (19)

Ten grams of (13) (0.038 moles) were dissolved in 60 ml of acetone and a molar excess of methyl iodide (10.8 g) was added. The solution was refluxed for two hours. The solvent was removed, leaving an orange oil: ^{1}H NMR ($CDCl_3$, $(CD_3)_2SO$) δ 9.7 (2 H, m), 9.0 (1 H, m), 8.5 (1 H, m), 4.8 (3 H, s), 4.4 (2 H, t), 0.95 (3 H, t); IR (NaCl) ν (C-H) 2944, (C=O) 1725, (-C-C(=O)O) 1297, (O-C-C) 1113, (γ CH) 741, (β ring) 658.

3-(β-Phenylethoxycarbonyl)-1-methylpyridinium Iodide/β-Phenethyl N-Methylnicotinate Iodide (20)

Ten grams of (14) (0.044 moles) were dissolved in 60 ml of acetone. A 1.0 M excess of methyl iodide (12.5 g) was added to the liquid and the system was allowed to reflux for two hours. The solvent was removed, yielding a solid which was recrystallized from acetone: ^{1}H NMR ($CDCl_3$, $(CD_3)_2SO$) δ 9.4 (2 H, m), 8.7 (1 H, m), 8.2 (1 H, m), 7.2 (5 H, s), 4.7 (3 H, s), 4.6 (2 H, t), 3.1 (2 H, t); IR (NaCl) ν (C-H) 2995 and 3023, (C=O) 1724, (-C-C(=O)O) 1290, (O-C-C) d 1135 and 1124, (γ CH) 731, (β ring) 657.

1,4-Dihydro-1-methyl-3-pyridinecarboxamide/1-Methyl-1,4-Dihydronicotinamide (21)

Four and six-tenths grams of sodium hydrogen carbonate ($NaHCO_3$) and 2.64 g of (6) (0.019 moles) were dissolved in 100 ml of water and cooled in an ice bath. To this stirring solution was added 6.96 grams of sodium dithionite ($Na_2S_2O_4$). A stream of nitrogen (N_2) covered the reaction mixture. After one hour, the reaction was stopped and the solution was extracted with several aliquots of $CHCl_3$. The $CHCl_3$ layer was removed under reduced pressure, yielding an orange oil. The oil was dissolved in a minimal amount of $CHCl_3$ and tritrated with petroleum ether. From this, an oil appeared and this was removed and dried in vacuo: ^1H NMR (D_2O) δ 6.9 (1 H, s), 5.7 (1 H, d), 4.8 (1 H, m), 3.2 (2 H, s), 3.0 (3 H, s); UV 355 nm λ_{max}; Elemental analysis calculated %: C, 60.87; H, 7.25; N, 20.20. Found %: C, 60.92; H, 7.29; N, 20.36 ($C_7H_{10}N_2O$).

1,4-Dihydro-1-(phenylmethyl)-3-pyridinecarboxamide/1-Benzyl-1,4-dihydronicotinamide (22)

To 100 ml of water were added 4.6 g of $NaHCO_3$ and 2.93 g of (7) (0.013 moles). The solution was cooled and 6.96 g of $Na_2S_2O_4$ were added. After two hours of stirring under N_2, a precipitate formed. The solution was filtered. The solid was recrystallized from aqueous methanol, giving lemon-yellow needles: ^1H NMR (D_2O) δ 7.2 (6 H, s), 7.1 (1 H, s), 5.7 (1 H, m), 4.7 (1 H, m), 4.2 (2 H, s), 3.1 (2 H, m), in $CDCl_3$ two protons at 5.8 δ appear; UV 357 nm λ_{max}; Elemental analysis calculated %: C, 72.29; H, 6.58; N, 12.97. Found %: C, 72.09; H, 6.60; N, 12.84 ($C_{13}H_{14}N_2O$).

1,4-Dihydro-1-methyl-3-pyridinecarboxylic Acid Ethyl Ester/Ethyl 1,4-Dihydro-N-methylnicotinate (23)

Four and six-tenths grams of $NaHCO_3$ and 2.75 g of (15) (0.016 moles) were dissolved in 100 ml of water and cooled in an ice bath. To this stirring solution was slowly added 6.96 g of $Na_2S_2O_4$. Two hundred milliliters

of ethyl ether were then added so that the dihydro would be extracted upon formation. This two-phase system avoided tetrahydropyridine production. The reaction proceeded for one hour under nitrogen. The ether layer was removed and the aqueous layer extracted. The combined ether fractions were dried over Na_2SO_4 and the solvent removed under reduced pressure. The resulting orange-red oil was dried in vacuo: ^1H NMR ($CDCl_3$) δ 6.9 (1 H, d), 5.6 (1 H, m), 4.8 (1 H, m), 4.1 (2 H, g), 3.1 (2 H, m), 2.9 (3 H, s), 1.2 (3 H, t); UV 358 nm λ_{max}; Elemental analysis calculated %: C, 64.67; H, 8.17; N, 8.43. Found %: C, 64.65; H, 7.88; N, 8.34 ($C_9H_{13}NO_2$).

1,4-Dihydro-1-methyl-3-pyridinecarboxylic Acid Butyl Ester/Butyl 1,4-Dihydro-N-methylnicotinate (24)

A solution of 4.6 g of $NaHCO_3$ and 3.12 g of (16) (0.016 moles) was prepared in 100 ml of water. The solution was cooled and 6.96 g of $Na_2S_2O_4$ were added. Two hundred milliliters of ethyl ether were added and this mixture stirred under nitrogen for one hour. The ether layer was removed, dried with Na_2SO_4 and reduced in volume. The resulting oil was dried in vacuo: ^1H NMR ($CDCl_3$) δ 6.9 (1 H, s), 5.6 (1 H, m), 4.7 (1 H, m), 4.1 (2 H, t), 3.1 (2 H, m), 2.9 (3 H, s), 0.9 (3 H, t); UV 358 nm λ_{max}; Elemental analysis calculated %: C, 67.69; H, 9.07; N, 7.23. Found %: C, 67.58; H, 8.82; N, 7.09 ($C_{11}H_{17}NO_2$).

1,4-Dihydro-1-methyl-3-pyridinecarboxylic Acid Hexyl Ester/Hexyl 1,4-Dihydro-N-methylnicotinate (25)

A solution of 4.6 g of $NaHCO_3$ and 3.57 g of (17) (0.016 moles) was prepared in 100 ml of water. The solution was cooled and 6.96 g of $Na_2S_2O_4$ were added. Two hundred milliliters of ethyl ether were added and the mixture stirred under nitrogen for one hour. The ether layer was separated, dried and reduced in volume. The dried oil was orange in color: ^1H NMR ($CDCl_3$) δ 6.8 (1 H, s), 5.5 (1 H, m), 4.6 (1 H, m), 4.0 (2 H, t),

3.1 (2 H, m), 3.0 (3 H, s), 0.9 (3 H, t); UV 359 nm λ_{max}; Elemental analysis calculated %: C, 69.96; H, 9.73; N, 6.32. Found %: C, 69.82; H, 9.46; N, 6.28 ($C_{13}H_{21}NO_2$).

1,4-Dihydro-1-methyl-3-pyridinecarboxylic Acid Octyl Ester/Octyl 1,4-Dihydro-N-methylnicotinate (26)

A solution of 4.6 g of $NaHCO_3$ and 4.02 g of (18) (0.016 moles) was prepared in 5% aqueous methanol. The solution was cooled and 6.96 g of $Na_2S_2O_4$ were slowly added. Two hundred milliliters of ethyl ether were added and the mixture stirred under nitrogen for one hour. The ether layer was separated, dried and reduced in volume. The oil was dried in vacuo: ^1H NMR ($CDCl_3$) δ 6.9 (1 H, m), 5.6 (1 H, m), 4.7 (1 H, m), 4.0 (2 H, t), 3.0 (2 H, m), 2.9 (3 H, s), 0.9 (3 H, t); UV 358 nm λ_{max}; Elemental analysis calculated %: C, 70.69; H, 9.97; N, 5.50. Found %: C, 70.76; H, 9.68; N, 5.88 ($C_{15}H_{25}NO_2 \cdot \frac{1}{5}H_2O$).

1,4-Dihydro-1-methyl-3-pyridinecarboxylic Acid Decyl Ester/Decyl 1,4-Dihydro-N-methylnicotinate (27)

A solution of 4.6 g of $NaHCO_3$ and 4.46 g of (19) (0.016 moles) was prepared in 5% aqueous methanol. The solution was cooled and 6.96 g of $Na_2S_2O_4$ were slowly added. To this solution was added 200 ml of ethyl ether and the mixture stirred under N_2 for one hour. The ether layer was separated, dried and reduced in volume. The dried oil was orange in color: ^1H NMR ($CDCl_3$) δ 6.9 (1 H, m), 5.6 (1 H, m), 4.7 (1 H, m), 4.0 (2 H, t), 3.0 (2 H, m), 2.9 (3 H, s), 0.9 (3 H, t); UV 359 nm λ_{max}; Elemental analysis calculated %: C, 73.12; H, 10.39; N, 5.02. Found %: C, 73.16; H, 10.48; N, 5.03 ($C_{17}H_{29}NO_2$).

1,4-Dihydro-1-methyl-3-pyridinecarboxylic Acid β-Phenylethyl Ester/β-Phenethyl 1,4-Dihydro-N-methylnicotinate (28)

Four and six-tenths grams of $NaHCO_3$ and 3.89 g of (20) (0.016 moles) was dissolved in 100 ml of water. The solution was cooled and 6.96 g of

$Na_2S_2O_4$ were added. Two hundred milliliters of ethyl ether were added and the mixture stirred over N_2 for one hour. The ether layer was separated, dried and reduced in volume. The dried oil was orange in color: ^1H NMR ($CDCl_3$) δ 7.2 (5 H, s), 6.9 (1 H, m), 5.7 (1 H, m), 4.7 (1 H, m), 4.2 (2 H, t), 3.0 (2 H, m), 2.9 (2 H, t), 2.8 (3 H, s); UV 355 nm λ_{max}; Elemental analysis calculated %: C, 70.18; H, 6.63; N, 5.46. Found %: C, 70.27; H, 7.00; N, 5.10 ($C_{15}H_{17}NO_2 \cdot \frac{3}{4}H_2O$).

Characterization of Dihydroberberine

Distribution Coefficients

Fifty milliliters of a cold 1 x 10^{-4} M solution of berberine (1) in pH 7.4 buffer were partitioned against 50 ml of $CHCl_3$ or 50 ml of 1-octanol. The concentration of (1) was determined spectrophotometrically in the organic and aqueous layer. A stock solution of 2.7 x 10^{-3} M dihydroberberine hydrochloride (3) was made in methanol. An aliquot of this, sufficient to produce a 1 x 10^{-4} M solution, was pipetted into 50 ml of cold pH 7.4 buffer and extracted immediately with either $CHCl_3$ or 1-octanol. After allowing for oxidation, the concentration of (1) in the organic and aqueous layer was determined spectrophotometrically.

Potentiometric pK_a Determination of Dihydroberberine (2)

Due to the extreme water insolubility of (2) (< 3 µg/ml), all determinations were done in 25% methanolic solutions. A titration curve was generated by adding 10 µl aliquots of NaOH to a 1.0 mM solution of (3). The pK_a was determined by inspection of the titration curve. During the experiments, all solutions were covered with a stream of nitrogen.

Spectrophotometric pK_a Determination of Dihydroberberine (2)

The pK_a of (2) was determined by measuring the absorbance difference at 355 nm in basic, acidic, and buffered media. The relationship that allows this determination appears below:

$$pK_a = pH - \log \frac{\alpha_{obs} - \alpha_{HA}}{\alpha_{A^-} - \alpha_{obs}}$$

where α_{obs} is the absorbance in buffer, α_{A^-} is the absorbance in base and α_{HA} is the absorbance in acidic media.

Oxidation of Dihydroberberine (2) by Silver Nitrate

Two hundred milligrams of (2) were dissolved in 95% aqueous ethanol. Upon addition of a 10% solution of silver nitrate, a black precipitate formed. Centrifugation and analysis of the supernatant shows stoichiometric oxidation of (2).

Oxidation of Dihydroberberine (2) by Diphenylpicrylhydrazyl Free Radical (DPP·)

Two hundred milligrams of (2) were dissolved in acetonitrile. To this was added a solution of DPP· in acetonitrile which caused an immediate disappearance of the purple color due to DPP·. Ultraviolet analysis confirmed this oxidation.

Oxidation of Dihydroberberine (2) by Concentrated Hydrogen Peroxide

Two hundred milligrams of (2) were dissolved in 95% aqueous ethanol. A 30% solution of hydrogen peroxide was added and the system monitored by UV. The analysis demonstrated a rapid and complete oxidation of (2).

Oxidation of Dihydroberberine (2) in Buffers

The oxidation of (2) was determined by UV and an HPLC method. In the UV method, a solution of (3) was prepared and pipetted into buffers of various pH and at various temperatures. The changes of absorption at 460 nm were measured with time. Data acquisition was facilitated by an Apple II microprocessor and an enzyme kinetic software package. In the HPLC method, two buffers, pH 5.8 and pH 7.4, were used. The samples were maintained at 37°C in a water bath. At certain times, 5 µl of the solulution were injected onto a µBondapak C_{18} reverse-phase column, and the

peak heights analyzed. The mobile phase was 60:40 acetonitrile: pH 6.2 phosphate buffer and the flow rate was 2 ml/min.

Quantitation of the Oxidation of Dihydroberberine (2) and Various Dihydropyridines (21) and (22) in Hydrogen Peroxide

Solutions of (3), (21) and (22) were prepared. An aliquot of these solutions was added to a standardized solution of hydrogen peroxide (H_2O_2) (0.18 M). The appearance of the 460 nm peak of (1) or the disappearance of the 359 nm peak of the dihydronicotinamides was measured. This determination was made using the enzyme kinetics software package.

Quantitation of the Oxidation of Dihydroberberine (21) and Various Dihydropyridines (22)-(28) in Plasma

Freshly drawn 80% human plasma was obtained from Civitan Regional Blood Center. The oxidation of (3) was determined by HPLC and the oxidation of (3), (22), (23), (24), (25), (26), (27), and (28) by UV. In the HPLC analysis, a solution of (3) was added to 80% plasma and maintained at 37°C. At certain times, 1.0 ml of plasma was removed and treated with 3 ml of acetonitrile. The solution was centrifuged and 5 µl of the supernatant was analyzed by a µBondapak C_{18} reverse-phase column with a mobile phase of 60:40 acetonitrile: pH 6.2 phosphate buffer. The peak heights were analyzed and concentrations obtained from a standard curve. In the UV method, 40% plasma was maintained at 37°C in a kinetic cell. A solution of either (3) or one of the various dihydronicotinates was added to this and the appearance of the 460 nm absorbance of (1) or disappearance of the 359 nm absorbance of dihydronicotinates was observed.

Quantitation of the Oxidation of Dihydroberberine (2) and Various Dihydropyridines (22)-(28) in Liver Homogenate

The determination of the rate of oxidation of (3) in a liver homogenate by an HPLC method and (3), (22), (23), (24), (25), (26), (27), and (28) by a UV method was performed. In the HPLC method, 14 g of fresh rat liver

were homogenized in 45 ml of cold phosphate-buffered saline. To this was added a solution of (3), and the system was maintained at 37.0°C. At various times, 1.0 ml of the solution was removed and the protein precipitated with 3 ml of acetonitrile. The sample was centrifuged and 5 µl of the supernatant analyzed using a µBondapak C_{18} reverse-phase column with a mobile phase of 60:40 acetonitrile: pH 6.2 phosphate buffer. The peak heights were analyzed and concentrations obtained from a standard curve. The UV method involved homogenizing 7 g of rat liver in 15 ml of pH 7.4 phosphate buffer and diluting the homogenate to 200 ml (3.5% w/v). The homogenate was centrifuged and the supernatant was used. To this was added a solution of (3) or one of the dihydropyridines. The appearance of the 460 nm peak of berberine or the disappearance of the 359 nm peak of the dihydronicotinate was then measured.

Quantitation of the Oxidation of Dihydroberberine (2) and Various Dihydropyridines (22)-(28) in Brain Homogenate

Again, both an HPLC and UV method were employed in the determination of the rate of oxidation of (3), (22), (23), (24), (25), (26), (27) and (28). In the HPLC method, a solution of (3) was added to a 20% brain homogenate in pH 7.4 phosphate buffer. The homogenate was maintained at 37°C in a water bath. At various times, 1.0 ml of the homogenate was mixed with 3 ml of acetonitrile. The sample was centrifuged and the supernatant analyzed by the same method used in the liver homogenates. In the UV method, 2 g of freshly obtained rat brain were homogenized in 33 ml of pH 7.4 phosphate buffer, yielding a 6.0% w/v homogenate. The homogenate was centrifuged and the supernatant was used in the determinations. To this was added a solution of (3) or one of the dihydronicotinates. The appearance of 460 nm absorption of (1) or disappearance of the 359 nm peak of the dihydronicotinate was measured.

In Vitro Distribution of Berberine (1) and Dihydroberberine (2) in Whole Blood

Solutions of (1) or (3) were added to 60 or 75 ml of freshly drawn heparinized sheep's blood maintained at 37°C. At various times, 4 ml of blood were withdrawn. The blood was centrifuged and the plasma removed. One milliliter of the plasma was treated with 9 ml of acetonitrile and the supernatant was analyzed spectrophotometrically. The entire volume of the packed red blood cells was treated with 8 ml of acetonitrile and centrifuged. The supernatant was again analyzed spectrophotometrically. A standard curve was obtained by preparing solutions of known concentration in plasma or packed red blood cells. Recovery from the red blood cells was 71.4%.

Effect of Glucose on the Distribution of Berberine (1) in Whole Blood

Glucose was added to a volume of blood so that a concentration of 200 mg% was obtained. The above procedure was then repeated.

Animal Studies

In Vivo Characterization of Berberine (1) and Dihydroberberine (2)

White Sprague-Dawley rats, who weighed between 200-250 g, were anesthetized intramuscularly with Inovar^{R} (0.13 ml/Kg). Injections were made intravenously into the external jugular vein. The doses used include 55 mg/Kg of (2) in dimethylsulfoxide (DMSO), 55 mg/Kg of (3) in 20-25% aqueous ethanol, 55 mg/Kg of (1) in DMSO or 35 mg/Kg of (1) in DMSO. At certain times after the injection, the chest cavities of the rats were opened, the vena cava severed and the heart perfused with normal saline. Afterwards, the animals were decapitated and the brains removed. In certain experiments, the lungs, liver, and kidneys were also excised. The organs were then homogenized in a minimal amount of water, usually 2 ml, and extracted with 8 ml of acetonitrile. A standard curve of (1) in the

organ homogenate was constructed. Analysis was performed by injecting 5 µl of the supernatant onto a µBondapak C_{18} reverse-phase column with a mobile phase of 60:40 acetonitrile: phosphate buffer. The flow was 2 ml/min. Under these conditions, the retention time of (1) was 3.8 min and (2), 9.3 min.

Slow Infusion of Dihydroberberine Hydrochloride (3)

Rats were anesthetized and prepared as above. A dose of 55 mg/Kg of (3) was prepared in a volume of 1.0 ml. The vehicle was 20% aqueous ethanol. This dose was infused into the external jugular vein over a period of either thirty or forty-five minutes. At the end of the perfusion, the animals were decapitated, their organs collected and analyzed.

Effect of 1-Methyl-1,4-dihydronicotinamide (21) on the Efflux of Berberine from the Brain

Rats were anesthetized and prepared as above. Animals were injected with 200 mg/Kg of (21) in aqueous ethanol intravenously. After fifteen minutes, the standard dose of 55 mg/Kg of (3) was given. The animals were sacrificed at various times after the injection, selected organs were removed and homogenized, and the samples analyzed by HPLC.

In Vivo Characterization of 1-Benzyl-1,4-dihydronicotinamide (22)

Rats were anesthetized and cut down as above. Doses between 60 mg/Kg and 400 mg/Kg of (22) were administered. At various times after the injection, the animals were perfused, decapitated, and the brains removed. The brains were then frozen in liquid nitrogen and stored at 0°C until they were analyzed, at which time the brains were thawed, homogenized in 2 ml of water, and extracted with 8 ml of acetonitrile.

Analysis was made by HPLC using a µBondapak C_{18} reverse-phase column and a mobile phase of 40:60 acetonitrile: 1×10^{-3} M sodium heptanesulfonate. The dihydronicotinamide had a retention time of 3.4 min and the

quaternary compound had one of 10.6 min at a flow rate of 2 ml/min. A standard curve was constructed in brain homogenates.

Intracerebral Ventricular (icv) Administration

Sprague-Dawley rats were anesthetized with 70 µl/100 g of pentobarbital sodium, and atropine sulfate (0.05 mg/Kg) was given, if necessary. Injections of (1), (6), and ^{3}H-inulin (29) were made into the lateral ventricles of rat brains. The injections were made with the aid of a stereotaxic instrument and the site of the injection was -0.4 mm anterior-posterior, 1.5 mm medial-lateral and -3.0 mm dorsal-ventral relative to the bregma. The dose of (1) infused was 50 µg and the infusion volume was between 3 and 5 µl. The vehicle was DMSO and the infusion rate was 5 µl/5 min. In several experiments, (1) was coinjected with 1000 µg of (6). In another set of experiments, a dose of 2.3 µCi of (29) was injected icv.

At specific times after the infusions, the animals were decapitated. The brain was homogenized in 1.0 ml of water and (1) extracted with 4 ml of acetonitrile. Analysis of (1) was by HPLC with a mobile phase consisting of 50:50 acetonitrile: pH 6.2 phosphate buffer. A µBondapak C_{18} reverse-phase column was used and a standard curve constructed using brain homogenates. For radionuclide analysis, the brains were homogenized in 8 ml of water. Twenty-five hundredths of a milliliter of this homogenate were added to 0.75 ml of Soluene^{R}. After the sample dissolved, 12 ml of the scintillation cocktail were added. The samples were counted for five minutes and disintegrations per minute (dpm) were obtained by using a standard quench curve.

Limited Metabolic Studies

Rats treated intravenously (iv) with 55 mg/Kg of (1) or 55 mg/Kg of (3) were housed in metabolic cages. Urine was collected and extracted with

$CHCl_3$. The aqueous layer was then extracted with 3-methyl-1-butanol. The organic layers were reduced to dryness and then reconstituted with a small volume (50 µl) of methanol. This residue was used for HPLC and TLC analysis. Five microliters of each sample were analyzed by HPLC. A µBondapak C_{18} reverse-phase column and a mobile phase of 50:50 acetonitrile: pH 6.2 phosphate buffer were used. The TLC analysis consisted of spotting 5 µl of each sample on an alumina plate and eluting the system with cyclohexane: chloroform: acetic acid 45:45:10 or methanol. The plates were developed with iodine vapor or iodoplatinate spray reagent.

Toxicity

White CD-1 mice were employed in this study, the average mass of which was 22.6 ± 2 g. The mice were segregated into groups of 10, and 8-10 groups were used in each study. The doses given were determined by preliminary studies in which the LD_0 and the LD_{100} were obtained using small groups of animals. The doses were then prepared in equal increments between the two extremes, but there was additional emphasis placed at the lower end of the curve. Since this study was concerned with acute toxicity, the animals were injected intraperitoneally, and the number dead recorded after twenty-four hours. The groups were, however, observed an additional forty-eight hours to ensure an accurate appraisal of acute toxicity. Food and water were given ad libitum. The injection volume was 75-100 µl. The data were analyzed by fitting them to a sigmoid curve and by the method of Probits.

Anticancer Activity

Male BDF mice (20.6 ± 0.3 g) were used in this study. A suspension of P388 lymphocytic leukemia cells was injected intraperitoneally (ip) (1×10^6 cells) or intracerebrally (2.5×10^5 cells). The survival time of animals treated with various doses of (1) or (3) compared to the controls

was recorded. Berberine or dihydroberberine hydrochloride were given ip 3 times a day on day 2, 6, and 10 in 0.5% carboxymethylcellulose.

CHAPTER 3

RESULTS AND DISCUSSION

Synthesis and Characterization of Dihydroberberine

The initial step in the application of the proposed drug delivery system to berberine (1) is the preparation of its dihydro adduct. The first synthesis of dihydroberberine (2) involved disproportionation of (1) in strong base and was performed by Gadamer in 1905.[157,158] The mechanism of this reaction involves nucleophilic attack of hydroxide to the carbon adjacent to the nitrogen resulting in the formation of a transient amino alcohol. This intermediate collapses to oxyberberine and dihydroberberine, presumably via a hydride transfer. Several other syntheses for (2) have appeared in the literature and these involve the direct reduction of (1) by zinc amalgam,[159,160] complex metal hydrides[161,162] or sodium borohydride.[163,164] Historically, (2) has been of interest because of its spectroscopic properties,[165-167] and as an intermediate in certain synthetic schemes.[164] The use of (2) as a drug or in a drug delivery system is novel to this thesis.

In the present work (1) was reduced, as shown in Figure 3-1, by sodium borohydride in dry pyridine. Spectroscopic analysis showed that the yellow crystalline material produced was (2). The UV, IR, and MS are shown in Figures 3-2, 3-3, and 3-4 respectively, and are consistent with the assigned structure.[167-169] The ^{1}H NMR is presented in Figure 3-5, and the proton assignments in Table 3-1. The ^{13}C NMR is shown in Figure 3-6, and the corresponding carbon assignments in Table 3-2. These assignments were made by comparing (2) to a number of model systems.[170] The synthesized

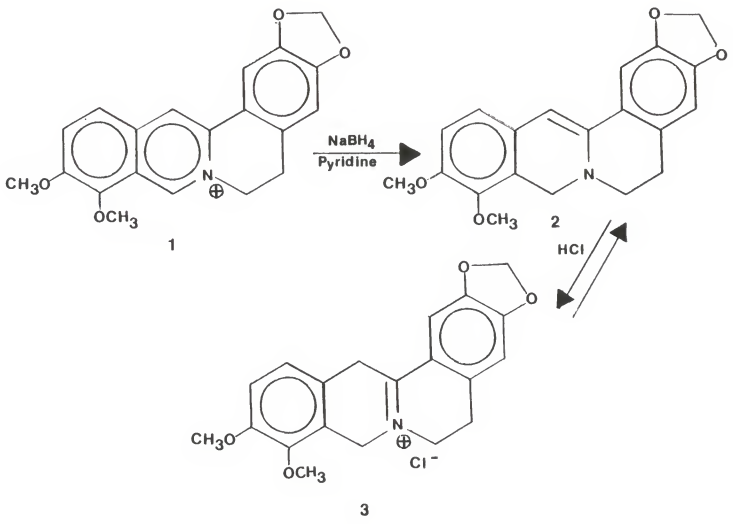


Figure 3-1. Synthesis of Dihydroberberine (2) and its Hydrochloride Salt (3).

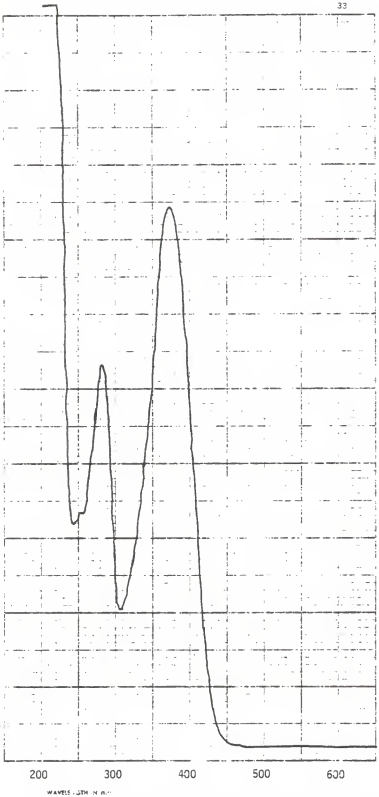


Figure 3-2. Ultraviolet Spectrum of Dihydroberberine (2) in 95% Ethanol

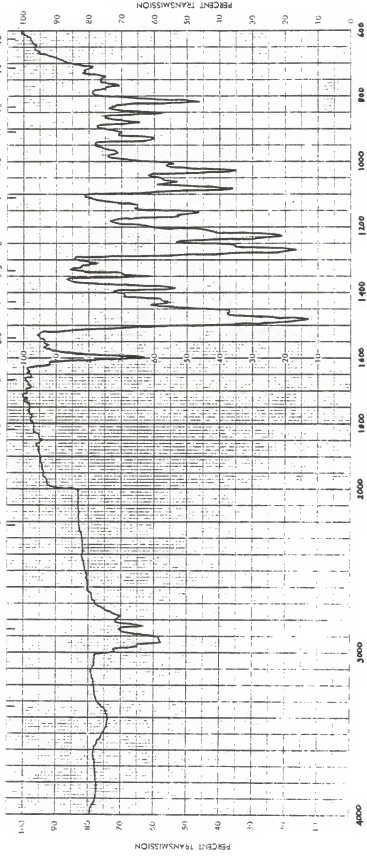


Figure 3-3 . Infrared Spectrum of Dihydroberberine (2) (KBr)

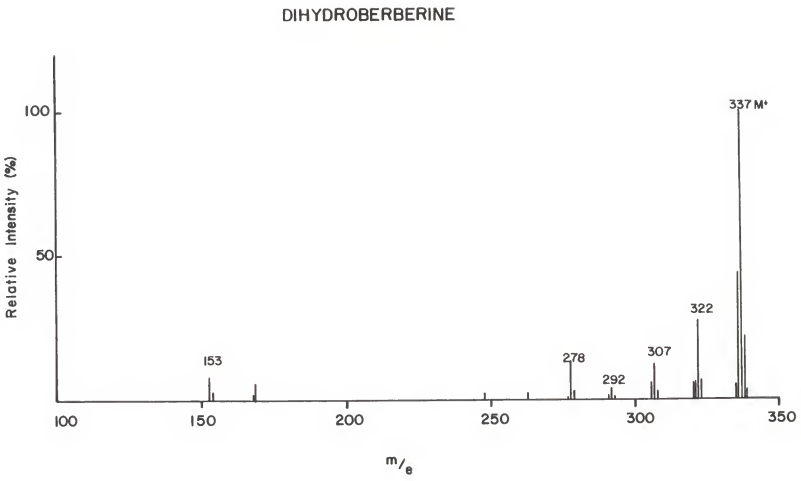


Figure 3-4. Mass Spectrum (70 eV, EI) of Dihydroberberine (2)

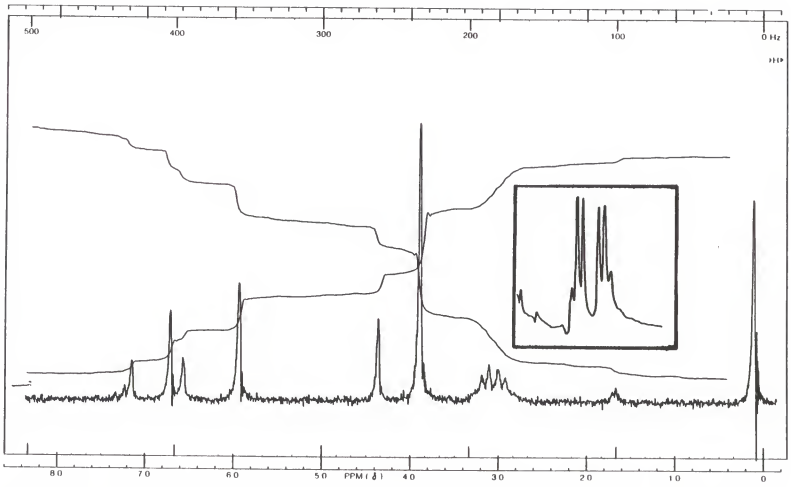
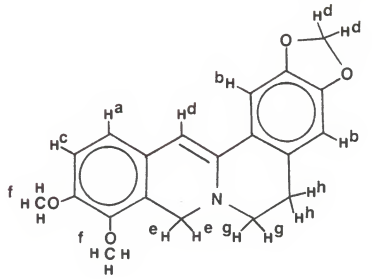


Figure 3-5. Proton Nuclear Magnetic Resonance Spectrum (60 MHz) of Dihydroberberine in $CDCl_3$. The Insert Represents the Region between 2.8δ and 3.4δ at 100 MHz

Table 3-1. Proton Assignments of the ^1H NMR of Dihydroberberine (2)



Proton	PPM (δ)
a	7.1
b	6.7
c	6.4
d	5.9
e	4.3
f	3.4
g	3.1
h	2.9

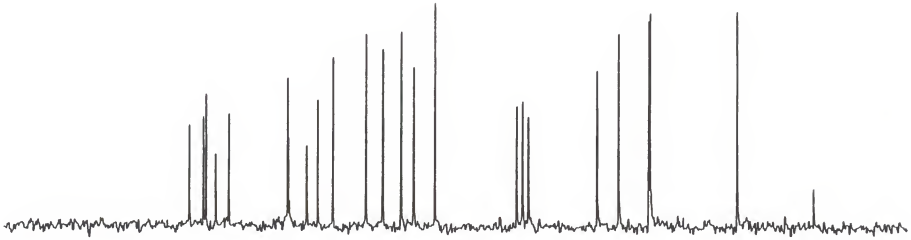
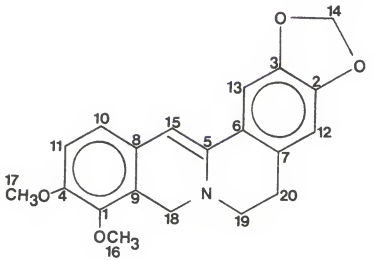


Figure 3-6. The ^{13}C Nuclear Magnetic Resonance Spectrum (100 MHz, $CDCl_3$) of Dihydroberberine (2)

Table 3-2. Carbon Assignments of the ^{13}C NMR of Dihydroberberine (2)



Carbon Number	PPM (δ)	Carbon Number	PPM (δ)
1	150.301	11	111.457
2	147.182	12	107.753
3	146.597	13	103.659
4	144.452	14	100.881
5	141.528	15	96.202
6	128.661	16	60.576
7	128.466	17	55.848
8	124.470	18	49.270
9	122.033	19	48.929
10	118.670	20	29.725

($CDCl_3$: 78.267, 77.040, 75.732)

material gave an elemental analysis in good agreement with that predicted for (2). Also, the reduced material reacted rapidly with such oxidizing agents as hydrogen peroxide, silver nitrate, and 1,1-diphenyl-2-picryl-hydrazyl free radical (DPP·) to yield (1). These data support the successful synthesis of (2).

The hydrochloride of dihydroberberine (3) was synthesized, as illustrated in Figure 3-1, by treating a concentrated solution of (2), in methylene chloride with dry hydrogen chloride (HCl) gas. The hydrochloride reverts to (2) at pH above 7.0. Analysis of the regenerated material demonstrated no addition of HCl or any other nucleophile to the molecule. This addition is known to occur with several dihydropyridines. The hydrochloride is many times more soluble in aqueous solutions than the free base.

If (2) is to be successful in a drug delivery system described in Figure 1-3, it should demonstrate a greater lipophilicity than (1). Dihydroberberine is expected to be less polar than (1) because of the loss of the positive charge. To investigate the relative lipid solubility of (2) compared to (1), the two compounds were extracted with organic solvents and their distribution (partition) coefficients compared.[171]

The two solvent systems which were used included an octanol-pH 7.4 phosphate buffer and a chloroform-pH 7.4 phosphate buffer. The alcohol-buffer system was chosen as one of the extracting systems because of its ability to, in some ways, mimic the partitioning of compounds in vivo. The data from Table 3-2 show that in both systems (2) has a high affinity for the organic phase while (1) exhibits a high affinity for the aqueous phase.

The chemistry of dihydroberberine is largely a result of its enamine character.[172] Dihydroberberine, like all enamines, is in equilibrium with

Table 3-3. Distribution Coefficients for Berberine (1) and Dihydroberberine Hydrochloride (3) in Chloroform/pH 7.4 Buffer and in 1-Octanol/pH 7.4 Buffer

Compound	Distribution Coefficient	
	Chloroform/pH 7.4 Buffer	1-Octanol/pH 7.4 Buffer
Berberine (1)	< 0.001	0.062
Dihydroberberine Hydrochloride (3)	5.33	2.59

its corresponding imine and this unusual situation allows enamines, dihydroberberine included, to be substrates in both nucleophilic and electrophilic reactions. This equilibrium tends to concentrate a negative charge on the carbon β to the nitrogen. Protonation usually occurs, for this reason, at the β-carbon rather than the nitrogen. At physiological pH, a portion of the dihydroberberine molecules will exist in a C-protonated state. The pK_a which is an indication of the degree of this ionization is important since only the unprotonated free base is available for diffusion across membranes. The pK_a of (2) was determined to be 6.80 ± 0.05 by both a spectrophotometric and potentiometric method. At a physiological pH of 7.4, a substantial portion of (2) will thus exist in the unionized, freely diffusable form. Because of the water insolubility of the dihydroberberine free base, all pK_a determinations were carried out in 25% methanolic solutions.

Demethylation of Berberine

A number of other synthetic schemes were explored in an attempt to obtain a method for preparing radiolabeled (1), should spectroscopic method prove too insensitive. A radiolabelled compound would also greatly expedite whole body distribution studies. In radiolabeling a compound, one of the important factors governing the selection of a synthetic route is yield. The scheme which was chosen involves pyrolyzing (1) at 200°C.[173,174] Berberine loses methyl chloride, as shown in Figure 3-7, to form the deep purple zwitterionic berberrubin. This method would allow the placement of either a ^{14}C or ^{3}H label at the nine position of (1) by reacting berberrubin with the appropriately tagged methyl iodide or methyl sulfate. Methylation of berberine with cold methyl iodide was performed to demonstrate the viability of the scheme.

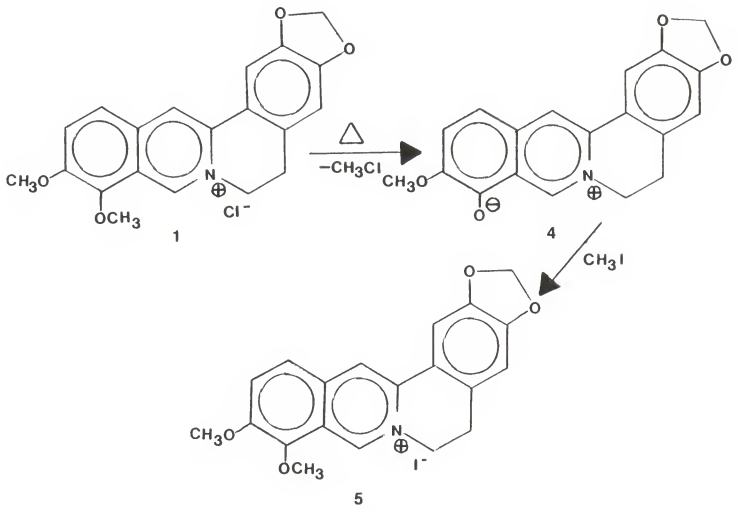


Figure 3-7. Demethylation of Berberine (1) and Methylation of Berberrubin (4).

Theoretical Studies on the Dihydropyridine ⇌ Pyridinium Redox System

If the drug delivery system described in Figure 1-3 is to be used to its potential, then, a knowledge of its basic chemistry is required. The reason for this is not only to understand the subtle chemistry inherent in this particular system, but also to allow prediction, extrapolation and generalization of the system to other examples. The ability to discern, for instance, those factors that add to or detract from the stability of a molecule would allow attenuation of a particular property by molecular manipulation. A thorough chemical knowledge of a particular compound would also allow an intelligent prediction as to its suitability to the scheme.

While a few ionization potentials have been measured, the general instability of dihydropyridines often precludes their investigation by experimental means. Because of this limitation, these compounds have lent themselves well to theoretical study. While the dihydronicotinamides, because of their biological relevance, have been the subject of copious reports, larger dihydro systems have received little attention.[175,176] In order to gain a greater chemical insight into the proposed drug delivery system in general, and the berberine (1) ⇌ dihydroberberine (2) system in particular, a theoretical investigation was undertaken.

Berberine is a rather large molecule and, as such, would be expected to present problems in terms of computational time. It must be remembered that 3N-6 (where N is the number of atoms) independent variables are required for a molecule and large molecules can easily cost $8000-$10000 in computer time. Because of this, and also in an attempt to generalize the calculations so that they would be applicable to a number of compounds, a model was developed for the (1) ⇌ (2) system. The compounds chosen as

the model, 3H,4H-dihydro-7,8-dihydroxybenzo[b]quinolizinium ion (30) and 3H,4H,6H-trihydro-7,8-dihydroxybenzo[b]quinolizine (31) along with their numbering protocol, are shown in Figure 3-8.

This quaternary and its dihydro adduct, while simpler than the (1) $\rightleftarrows$ (2) system, do not differ greatly in structure or in general chemistry. Compounds (30) and (31) have not been investigated and no mention of them has appeared in the literature. Some theoretical work on the unsubstituted benzo[b]quinolizinium ion and much on the isoquinoline molecule has been published. The benzo[b]quinolizinium ion was investigated by Galasso in 1968 and charge densities and π-bond orders were reported.[177] All of these reported studies have employed simplistic theoretical treatments such as PPP,[178] IOC-ω-technique,[179] HMO,[180] SSP,[181] and CNDO/2.[182] The corresponding dihydrocompounds have not been studied.

In order to investigate the isoquinoline model (30) $\rightleftarrows$ dihydroisoquinoline model (31) system, a MINDO/3 method was selected.[183] This approach is a semi-empirical self-consistent field molecular orbital method in which all valence electrons are treated. The MINDO/3 program is the culmination of the series MINDO,[184] MINDO/2,[185,186] and MINDO/2'.[187] This program was developed to solve chemical problems quickly and efficiently using a quantum mechanical framework. Unlike the methods of Pople, whose major aim was to reproduce nonempirical calculations, M.J.S. Dewar parametrized MINDO so that the system would produce useful and chemically accurate data. This program is extremely versatile and has been applied to a number of chemical problems including reactions. Only the most cursory details of MINDO/3 are appropriate here, as the subject is well reviewed elsewhere.[183,188]

In quantum mechanical approaches like MINDO, a molecular orbital is the result of the interaction of a wavefunction of an electron with nuclei and other electrons present in the molecule.[189] Mathematically, the

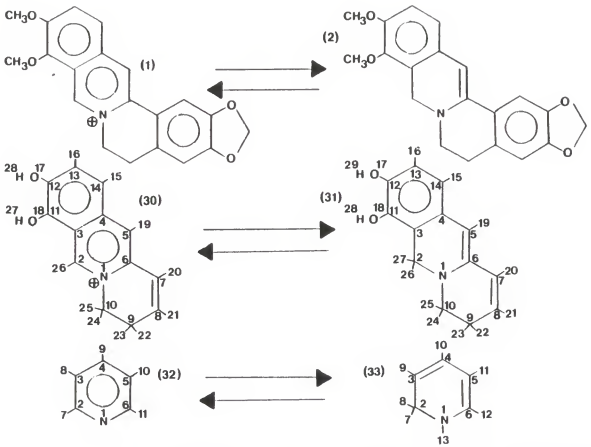


Figure 3-8. Structures and Salient Numbering Protocols for Berberine (1), Dihydroberberine (2), the Isoquinoline Model (30), the Dihydroisoquinoline Model (31), Pyridine (32), and 1,2-Dihydropyridine (33).

Hamiltonian for such a system consists of the kinetic energy term for the movement of the electrons and the potential energy terms for electron-nuclei attraction and electron-electron repulsion.

In systems with only one electron, e.g. H, H_2^+ or He^+, the differential equation which constitutes the Hamiltonian can be separated and exactly solved. If, however, more than one electron is present, the electrons interact and the differential equation is no longer separable or exactly solvable. Because of this problem, approximations and simplifications are incorporated into the Schrödinger equation. These include considering the molecular orbital (Ψ) as a linear combination of atomic orbitals (ϕ):[189]

$$\Psi = \sum_i C_i \phi_i$$

Additional simplifications are obtained by neglecting certain electron repulsion terms since these values are close to zero. These approximations allow the application of quantum mechanical approaches like MINDO to rather complex chemical problems without extremely large expenditures of capital or computer time.

The MINDO/3 program provides the following data: optimized geometries of the most stable ground state conformation at 25°C, the heat of formation (ΔH_f) in kcal/mole at 25°C of the optimized structures, the atomic charge distribution, the dipole moments, the total electronic energy, the vertical ionization potentials, the bond order matrix, and the eigen vectors and eigen values.

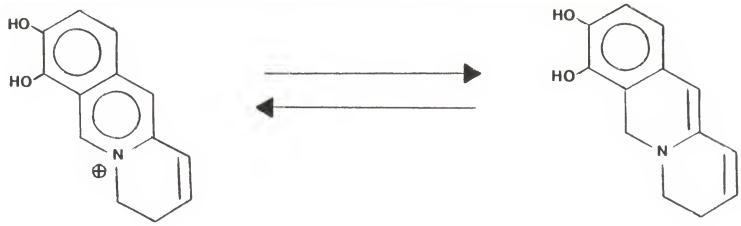
The eigen values and eigen vectors allow a thorough examination of the contributions of the individual atomic orbitals to the molecular orbital and thus of the electronic structure of the molecule. This can be indicative of many of the chemical proclivities of a molecule. The MINDO

approach allows one to examine compounds and reactions which are not approachable by conventional means. For example, the stability of very reactive species can be assayed, their structures determined, and qualitative aspects of their chemistry elucidated.

The input for the program includes, for each atom, the atomic number, the approximate bond distances between adjoining atoms, the approximate bond angle, and the approximate twist angle out of the plane. As previously mentioned, the number of parameters needed is 3N-6 where N is the number of atoms. Convergence is assumed when the difference between two successive calculations is less than 0.1 kcal/mole.

The two models (30) and (31) were analyzed and the results are presented in Table 3-4 through 3-8. The heat of formation calculated for (30) was 95.1 kcal/mole and that of (31) was -39.3 kcal/mole. While the use of ΔH_f for comparisons is restricted to structural isomers, the relative stability of a system can be calculated by comparing the differences in ΔH_f ($\Delta\Delta H_f$) from one system to another. The $\Delta\Delta H_f$ of the isoquinoline model (30) $\rightleftarrows$ dihydroisoquinoline model (31) pair is 134.4 kcal/mole. As shown in Table 3-9, this value is smaller than that obtained from simple dihydropyridine $\rightleftarrows$ pyridinium systems indicating a greater stability of (31) relative to simple dihydropyridines.[190] The stabilization of (31) indicates that it should be less reactive than simple dihydropyridines. The basis of this stabilization is derived from the extended aromatic conjugation of (31). Another contribution to the relative stabilization can be seen by an examination of the highest occupied molecular orbital (HOMO) which is represented in Figure 3-9 as a linear combination of atomic orbitals. In looking at the HOMO, only the magnitude of the coefficients is important since the sign simply represents the phase of the orbital. A

Table 3-4. The Heats of Formation, Vertical Ionization Potentials, and Dipole Moments of the Isoquinoline Model (30) and the Dihydroisoquinoline Model (31).



ISOQUINOLINE MODEL (30)		DIHYDROISOQUINOLINE MODEL (31)
95.1	HEAT OF FORMATION (kcal/mol)	-39.3
11.56(π)	VERTICAL I.P. (eV)	7.19(π-P_N)
12.94(π-P_N)		8.31(π)
		9.07(P_N)
		9.44(σ)
		9.58(σ)
15.43	DIPOLE MOMENT (Debye)	1.73

Table 3-5. Bond Lengths in Angstroms between Various Atoms of the Isoquinoline Model (30) and the Dihydroisoquinoline Model (31)

Isoquinoline Model (30)				Dihydroisoquinoline Model (31)			
Atom Number	Bond Length	Atom Number	Bond Length	Atom Number	Bond Length	Atom Number	Bond Length
1-2	1.342	13-14	1.389	1-2	1.451	13-14	1.397
2-3	1.433	14-4	1.433	2-3	1.517	11-18	1.321
3-4	1.467	11-18	1.321	3-4	1.445	12-17	1.337
4-5	1.444	12-17	1.337	4-5	1.468	2-27	1.132
5-6	1.388	2-26	1.115	5-6	1.370	2-26	1.130
6-1	1.415	5-19	1.110	6-1	1.401	5-19	1.110
6-7	1.480	7-20	1.103	6-7	1.485	7-20	1.103
7-8	1.345	8-21	1.106	7-8	1.343	8-21	1.106
8-9	1.487	9-22,23	1.118	8-9	1.489	9-23,22	1.118
9-10	1.518	10-24,25	1.123	9-10	1.521	10-24,25	1.123
10-1	1.480	13-16	1.106	10-1	1.452	13-16	1.106
3-11	1.467	14-15	1.104	3-11	1.444	14-15	1.104
11-12	1.418	18-27	0.952	11-12	1.432	18-28	0.951
12-13	1.433	17-28	0.950	12-13	1.417	17-29	0.951

Table 3-6. Bond Angles in Degrees between Various Atoms of the Isoquinoline Model (30) and the Dihydroisoquinoline Model (31)

Isoquinoline Model (30)				Dihydroisoquinoline Model (31)			
Atom Number	Bond Angle^0	Atom Number	Bond Angle^0	Atom Number	Bond Angle^0	Atom Number	Bond Angle^0
2-1-6	121.6	3-2-26	120.4	2-1-6	121.8	1-2-26	108.3
3-4-5	115.7	13-14-15	199.9	3-4-5	116.3	1-2-27	108.8
4-5-6	125.5	12-13-16	119.7	4-5-6	126.4	13-14-15	119.9
1-6-5	116.2	13-12-17	117.2	1-6-5	117.9	12-13-16	119.7
1-6-7	117.1	12-11-18	128.1	1-6-7	116.5	13-12-17	117.2
6-7-8	123.7	6-7-20	114.5	6-7-8	123.6	12-11-18	128.1
7-8-9	121.9	7-8-21	121.3	7-8-9	121.8	6-7-20	114.5
6-1-10	121.9	8-9-22	110.7	6-1-10	123.4	7-8-21	121.3
1-3-4	118.6	8-9-23	108.9	11-3-4	118.7	8-9-22	109.8
2-11-3	120.5	9-10-24	109.7	12-11-3	120.4	8-9-23	109.0
1-12-13	119.1	9-10-25	109.8	11-12-13	119.3	9-10-24	108.0
2-13-14	121.8	12-17-28	114.3	12-13-14	121.1	9-10-25	108.6
4-5-19	117.8	11-18-27	114.3	4-5-19	116.3	12-17-29	113.9
						11-18-28	113.9

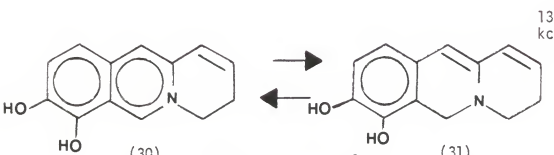
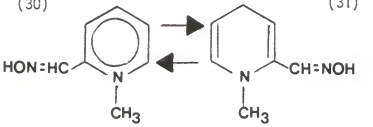
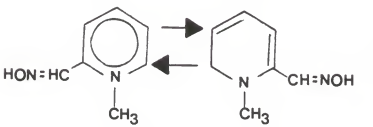
Table 3-7. Charge Density at Various Atoms of the Isoquinoline Model (30) and the Dihydroisoquinoline Model (31)

Isoquinoline Model (30)				Dihydroisoquinoline Model (31)			
Atom Number	Charge Density	Atom Number	Charge Density	Atom Number	Charge Density	Atom Number	Charge Density
1	+0.0779	15	+0.0432	1	-0.1534	15	+0.0044
2	+0.1827	16	+0.0533	2	+0.2443	16	+0.0104
3	-0.1151	17	-0.4266	3	-0.1434	17	-0.4508
4	+0.1029	18	-0.4276	4	+0.0979	18	-0.4283
5	-0.0429	19	+0.0512	5	-0.1597	19	+0.0055
6	+0.1080	20	+0.0514	6	+0.1737	20	+0.0081
7	-0.0446	21	+0.0491	7	-0.0311	21	+0.0001
8	+0.0359	22	+0.0221	8	+0.0024	22	-0.0257
9	+0.0546	23	+0.0128	9	+0.0724	23	-0.0280
10	+0.1104	24	-0.0011	10	+0.1777	24	-0.0610
11	+0.3261	25	+0.0010	11	+0.2993	25	-0.0472
12	+0.2019	26	+0.0429	12	+0.1775	26	-0.0663
13	+0.0340	27	+0.2847	13	-0.0206	27	-0.0788
14	-0.0582	28	+0.2699	14	-0.0652	28	+0.2463
						29	+0.2394

Table 3-8. Dihedral Angles between Various Atoms of the Isoquinoline Model (30) and the Dihydroisoquinoline Model (31)

Isoquinoline Model (30)				Dihydroisoquinoline Model (31)			
Atom Number	Dihedral Angle, θ	Atom Number	Dihedral Angle, θ	Atom Number	Dihedral Angle, θ	Atom Number	Dihedral Angle, θ
14-13-12-11	-1.1	11-12-13-16	180.0	14-13-12-11	0.0	14-13-12-17	180.0
13-12-11-3	0.0	14-13-12-17	180.0	13-12-11-3	0.0	13-12-11-18	180.0
12-11-3-4	0.0	13-12-11-18	180.0	12-11-3-4	0.0	8-6-7-20	180.0
11-3-4-5	179.7	1-6-7-20	176.9	11-3-4-5	180.0	9-7-8-21	180.0
3-4-5-6	0.5	6-7-8-21	181.0	3-4-5-6	3.1	10-8-9-22	123.8
4-5-6-1	1.6	10-8-9-22	123.8	4-5-6-1	1.3	10-8-9-23	236.1
5-6-1-2	-2.7	10-8-9-23	236.1	5-6-1-2	-9.1	1-9-10-24	122.6
2-1-6-7	177.4	1-9-10-24	122.6	5-1-6-7	180.0	1-9-10-25	234.9
1-6-7-8	-3.6	1-9-10-25	234.9	1-6-7-8	1.6	6-4-5-19	180.0
6-7-8-9	0.3	3-4-5-19	180.0	6-7-8-9	0.1	13-12-17-29	92.0
2-6-1-10	179.1	13-12-17-28	87.9	2-6-1-10	180.2	3-11-18-28	180.0
12-13-14-15	180.0	3-11-18-27	174.7	12-13-14-15	180.0	3-1-2-26	125.4
		4-3-2-26	179.4	11-12-13-16	180.0	3-1-2-27	235.0

Table 3-9. Differences in the Heats of Formations ($\Delta\Delta H_f$) of (30) ⇄ (31), 2-PAM ⇄ 1,4-Dihydro-2-PAM and 2-PAM ⇄ 1,6-Dihydro-2-PAM

	$\lvert\Delta\Delta H_f\rvert$
	134.40 kcal/mole
	Isomer Anti 140.03 Syn 145.15
2-PAM 1,4-dihydro-2-PAM	
	Anti 134.42 Syn 139.20
2-PAM 1,6-dihydro-2-PAM	

$$\psi_{HOMO}^{(31)} = 0.449\ p_z(N_1) + 0.042\ p_z(C_2) - 0.221\ p_z(C_3) - 0.247\ p_z(C_4)$$

$$+ 0.479\ p_z(C_5) + 0.291\ p_z(C_6) - 0.074\ p_z(C_7) - 0.174\ p_z(C_8) + 0.013\ p_z(C_9)$$

$$+ 0.018\ p_z(C_{10}) + 0.130\ p_z(C_{11}) + 0.343\ p_z(C_{12}) + 0.106\ p_z(C_{13}) - 0.284\ p_z(C_{14})$$

$$+ 0.005\ s(H_{15}) + 0.006\ s(H_{16}) - 0.085\ p_z(O_{17}) - 0.079\ p_z(O_{18}) - 0.008\ s(H_{19})$$

$$+ 0.008\ s(H_{20}) - 0.010\ s(H_{21}) + 0.041\ s(H_{22}) - 0.034\ s(H_{23}) + 0.113\ s(H_{24})$$

$$- 0.082\ s(H_{25}) + 0.138\ s(H_{26}) - 0.179\ s(H_{27}) - 0.005\ s(H_{28}) - 0.092\ s(H_{29})$$

Figure 3-9. The Highest Occupied Molecular Orbital of the Dihydroisoquinoline Model (31)

relatively large contribution to the HOMO is made by the methylene hydrogens 27, 28 and 24, 25 and the nonaromatic carbon, C_2. This phenomenon is termed hyperconjugation. Hyperconjugation also occurs in simple 1,2- and 1,4-dihydropyridines but, because of the additional methylene interactions, the effect is slightly larger in the case of (31).[190] The large contribution to the HOMO by the nitrogen lone pair is also noted.

The vertical ionization potentials (IP) of (30) and (31) were calculated using Koopman's Theorem which simply states that the ionization potential is the negative of orbital energy. The calculated values are presented in Table 3-4 and are 11.56 eV for (30) and 7.19 eV for (31). In addition, the character of the orbital which loses the electron can be obtained by examination of the HOMO and, for (30), a π-type system is involved while for (31) a mixed π-P_N type orbital occurs. These values are similar to values obtained from other systems and are consistent with the molecular structures.[190] The dipole moments calculated for (30) and (31) also appear in Table 3-4 and, again, are consistent with the molecular structure.

It is instructive to compare changes that occur upon reduction of simple dihydropyridines to those that occur upon reduction of (31). This comparison demonstrates the similarity in chemistry between simple and more highly conjugated dihydropyridines and also demonstrates the extreme usefulness of the computational method. Tables 3-10 to 3-12 are comparisons of the pyridine (32) $\rightleftarrows$ dihydropyridine (33) system and the pyridinium nucleus of (30) $\rightleftarrows$ (31) system. The values for the (32) $\rightleftarrows$ (33) system were calculated by a MINDO procedure by Bodor and Pearlman in 1976.[190] The numbering of these various compounds appears in Figure 3-8.

Table 3-10 shows the bond lengths in the isoquinoline model system, (30) $\rightleftarrows$ (31), and the simple pyridine system, (32) $\rightleftarrows$ (33). Upon reduction

Table 3-10. A Comparison of Bond Lengths in Angstroms of the Pyridine (32) ⇄ 1,2-Dihydropyridine (33) System and the Isoquinoline (30) ⇄ Dihydroisoquinoline (31) Model System

Pyridine (32)		Dihydropyridine (33)		Isoquinoline Model (30)		Dihydroisoquinoline Model (31)	
Atom Number	Bond Length	Atom Number	Bond Length	Atom Number	Bond Length	Atom Number	Bond Length
1-2	1.336	1-2	1.432	1-2	1.342	1-2	1.451
2-3	1.402	2-3	1.492	2-3	1.433	2-5	1.517
3-4	1.406	3-4	1.357	3-4	1.467	3-4	1.445
4-5	1.406	4-5	1.453	4-5	1.444	4-5	1.468
5-6	1.402	5-6	1.351	5-6	1.388	5-6	1.370
6-1	1.336	6-1	1.363	6-1	1.415	6-1	1.401
7-2	1.107	7-2	1.134	2-26	1.115	2-26	1.130
		8-2	1.134			2-27	1.132
8-3	1.105	9-3	1.105	3-11	1.467	3-11	1.444
9-4	1.114	10-4	1.105	4-14	1.433		
		5-11	1.101	5-19	1.110	5-19	1.110
		6-12	1.113	6-8	1.480	6-7	1.485

of (30), the bond connecting carbons 5-6 shortens, indicating a greater double bond character at this location, while the bonds between carbons 2-3 and between the carbon and nitrogen at position 1-2 lengthen, indicating an increased single bond character at these locations. This correlates well with the structural formalism and also mirrors those changes that occur in the reduction of (32) to (33). A similar study of bond angles is presented in Table 3-11.

The charge densities at specific atoms can be indicative of the type of chemistry that a compound undergoes. A study of the charge densities of atoms in (30) ⇄ (31) is presented in Table 3-7 and a comparison of this system to the (32) ⇄ (33) system appears in Table 3-12. The charge densities of the pyridinium nucleus of (30) reveal the most highly charged deficient center is at carbon C_2. One would expect nucleophilic attack at this electropositive position and, in fact, this is what is observed. These observations can be extended to berberine (1) since it is known that hydroxide, hydride, and acetonide attack (1) at this position. In general, nucleophiles attack pyridines at the carbon adjacent to the nitrogen.[172] The charge densities of the atoms in (31) indicate a highly electronegative center at C_5. One would therefore expect protonation and electrophilic attack at this location. This, again, is borne out experimentally. In the case of dihydroberberine (2), protonation as well as alkylation occurs here and, in general, this type of reaction is well known in the chemistry of enamines.[191-193] These properties and trends are also seen in the (32) ⇄ (33) pair.

The planarity of a pharmacologically active aromatic molecule, especially antineoplastic agents, is an extremely important parameter and many correlations between activity and toxicity and planarity have been made.

Table 3-11. A Comparison of the Bond Angles in Degrees of the Pyridine (32) ⇄ 1,2-Dihydropyridine (33) System and the Isoquinoline (30) ⇄ Dihydroisoquinoline (31) Model System

Pyridine (32)		Dihydropyridine (33)		Isoquinoline Model (30)		Dihydroisoquinoline Model (31)	
Atom Number	Bond Angle^0	Atom Number	Bond Angle^0	Atom Number	Bond Angle^0	Atom Number	Bond Angle^0
2-1-6	119.8	2-1-6	124.0	2-1-6	121.6	2-1-6	121.8
3-4-5	119.8	3-4-5	117.9	3-4-5	115.7	3-4-5	116.3
2-3-4	118.2	4-5-6	120.1	4-5-6	125.5	4-5-6	126.4
1-2-3	122.0	1-6-5	121.4	1-6-5	116.2	1-6-5	117.9
8-3-2	120.6	6-1-13	119.0	6-1-10	121.9	6-1-10	123.4
7-2-3	120.6	1-2-0	123.2	3-2-26	120.4	1-2-26	108.3
						1-2-27	108.8
3-4-9	120.1	6-5-11	119.9	4-5-19	117.8	4-5-19	116.3
		12-6-5	122.9	1-6-7	117.1	1-6-7	116.5
		10-4-5	119.6	6-7-8	123.7	6-7-8	123.6
		9-3-4	121.8	11-3-4	118.6	11-3-4	118.7
				9-10-24	109.7	9-10-24	108.0
				9-10-25	109.8	9-10-25	108.6

Table 3-12. A Comparison of the Atomic Charge Densities of the Pyridine (32) ⇄ 1,2-Dihydropyridine (33) System and the Isoquinoline (30) ⇄ Dihydroisoquinoline (31) Model System

Pyridine (32)		Dihydropyridine (33)		Isoquinoline Model (30)		Dihydroisoquinoline Model (31)	
Atom Number	Charge Density	Atom Number	Charge Density	Atom Number	Charge Density	Atom Number	Charge Density
1	-0.1641	1	-0.1174	1	+0.0779	1	-0.1534
2	+0.1351	2	+0.2577	2	+0.1827	2	+0.2443
3	-0.0659	3	-0.1162	3	-0.1151	3	-0.1434
4	+0.0732	4	+0.0911	4	+0.1029	4	+0.0979
5	-0.0657	5	-0.1678	5	-0.0429	5	-0.1597
6	+0.1348	6	+0.1670	6	+0.1080	6	+0.1737
7	-0.0151	7	-0.0903	26	+0.0429	26	-0.6663
		8	-0.0899			27	-0.0788
8	+0.0005	9	+0.0083	11	+0.3261	11	+0.2993
9	-0.0182	10	+0.0101	14	-0.0582	14	-0.0562
10	+0.0001	11	+0.0184	19	+0.0512	19	+0.0055
11	-0.0147	12	-0.0173	7	-.0446	7	-0.0311
		13	+0.0663	10	+0.1104	10	+0.1777

This characteristic is accessible by MINDO/3. The program calculates a dihedral angle which is a measure of the deviation from planarity of a molecule. Pyridine (32) is planar and 1,2-dihydropyridine (33) is planar within one degree. The dihedral angles of (30) $\rightleftarrows$ (31) are shown in Table 3-8. In the case of (30), the overall deviation from planarity is less than 3.6°. In berberine (1), which has an additional benzene ring annulated at the C_7-C_8 position, this difference should be less because of the planarizing effects of the added aromatic system. This would tend to cast doubt on the proposal that the reason berberine does not intercalate into deoxyribonucleic acid (DNA), as well as totally aromatic molecules such as coralyne, is due to the buckling of the C ring.[135,155] This nonplanarity is attributed to the partial saturation of that ring. The present study, however, indicates that this deviation is slight and probably plays a minor role in attenuation of the action of (1).

A more plausible reason for the lower intercalative ability of (1) is because of lower electronic interactions. When a molecule intercalates into DNA, there exists an electronic interaction between the base pairs of DNA and the π-cloud of the intercalating aromatic compound. The greater the stabilization of this complex, the greater is the DNA-molecular interaction. In berberine, there is a partial destruction of the aromatic system which lowers any electronic interaction. The hydrogens added to the C-ring act to increase the effective thickness of the molecule and this may play a role in decreasing macromolecular complexation. These structural concerns are apparent in Figures 3-10 and 3-11, which are the fully optimized structure for (30).

The dihydro model (31) contains one more sp^3 center than does (30), and this has a slight deplanarizing effect with the molecule twisting 9.1°

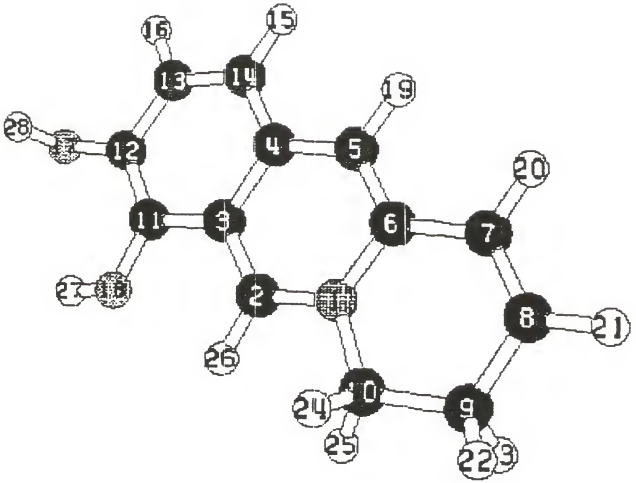


Figure 3-10. A Computer-assisted Drawing of the Most Stable Conformation of the Isoquinoline Model (30) at 25°C

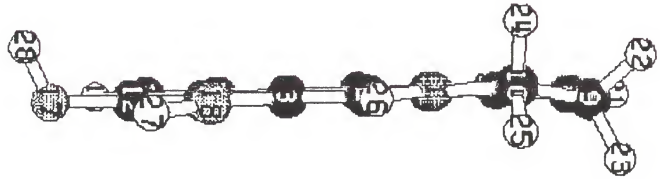


Figure 3-11. A Computer-assisted Drawing of the Most Stable Conformation of the Isoquinoline Model (30) at 25°C. This View is Oriented so that the Interatomic Axis between Atoms 26 and 2 is Perpendicular to the Plane of the Page

out of the plane. In applying the same considerations to this molecule as to (30), one would expect that this system would interact less with DNA than would (30). This is due to the further destruction of the aromatic nucleus resulting not only in a more nonplanar structure, but also in a structure with a lower propensity to interact electronically with DNA. The loss of the positive charge should also reduce macromolecular intercalation because of the lowered coulombic interaction between (31) and the anionic phosphate backbone of DNA.[156] In extending these results to dihydroberberine (2), one would predict a lower cytotoxicity and, therefore, toxicity of (2) relative to (1). The optimized structure of (31) at 25°C is presented in Figures 3-12 and 3-13.

To summarize, the MINDO/3 calculation predicts the dihydro model (31) and, presumably, (2) to be more stable than simple dihydropyridines because of extended conjugation and hyperconjugation. The model (30) is predicted to undergo nucleophilic attack at the carbon adjacent to the nitrogen, and protonation and electrophilic reaction at the carbon β to the nitrogen of the enamine. The calculations show localization of double and single bonds on the reduction of (30) to (31). These data are in good agreement with what is known about chemistry of this genre of compounds. The calculation suggests that the reason berberine does not intercalate as well as totally aromatic compounds is not because of steric problems associated with the carbon skeleton but, rather, electronic differences and the steric effects of added hydrogens and, finally, MINDO/3 predicts that (31) and presumably, (2) are less toxic than (30) and (1). These results should be applicable not only to the (1) $\rightleftarrows$ (2) system, but other conjugated dihydropyridine systems as well.

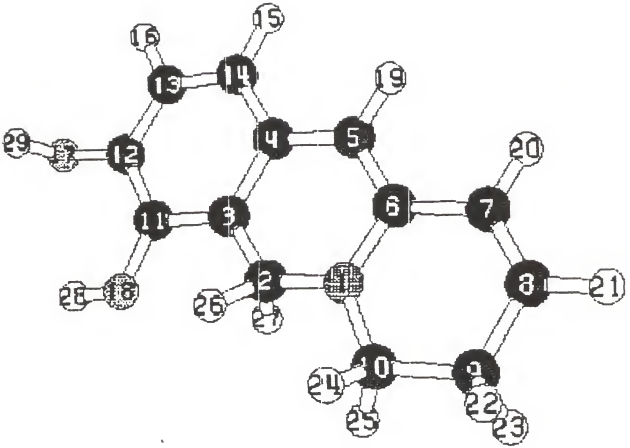


Figure 3-12. A Computer-assisted Drawing of the Most Stable Conformation of the 1,2-Dihydroisoquinoline Model (31) at 25°C

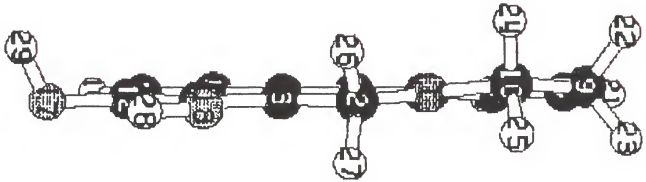


Figure 3-13. A Computer-assisted Drawing of the Most Stable Conformation of the 1,2-Dihydroisoquinoline Model (31) at 25°C. This View is Oriented so that an Imaginary Axis between Atoms 2 and 5 is Perpendicular to the Plane of the Page

Further Studies on the Biological and Chemical Properties of Dihydroberberine

The rate and nature of the oxidation of the dihydropyridines included in the delivery scheme is important to the proper functioning of the system. A dihydropyridine must be stable enough to be formulated and stored. The *in vivo* rate of oxidation must, however, be rapid enough to efficiently transform the delivering species and thereby avoid competing metabolisms.

The rate of oxidation of dihydroberberine (2) was therefore studied in a variety of media, and in a number of different situations. One problem which hampered these determinations was the extreme water insolubility of the free base. The rate of oxidation of (2) was determined by both HPLC and UV methods. The UV procedure involved measuring the appearance of the 460 nm absorbance of berberine (1) with time, while the HPLC determinations were made by calculating the appearance of the absorbance due to (1) or disappearance of the absorbance due to (2). In most cases agreement between the two methods was good. In all determinations the spectrum of (1) showed no change within the timeframe of the experiment. Initial oxidation studies were performed in areated buffer. At a pH of 7.4, (2) oxidized very rapidly and erratically. Buffers of lower pH were then used to partially stabilize (2) by shifting the equilibrium in favor of the hydrochloride in an effort to yield a more reproducible system. This shift reduces the electronic density at the nitrogen, and precludes the participation of the nitrogen lone pair in oxidative reactions.[172] The lower pH also greatly facilitates solubilization. The rate of oxidation of (2) at a pH of 5.8 is shown in Table 3-13, and the spectral changes that are characteristic of this oxidation are shown in Figure 3-14. Although the correlation coefficients are not good, second order kinetics are indicated with a calculated second-order rate constant of 44.4 ± 0.53

Table 3-13. The Rate of Oxidation of Dihydroberberine in Various Media

Medium (°C)	Pseudo First Order Rate Constant	Correlation	Concentration (M)	Second Order Rate Constant
pH 5.8 Buffer (37°C)	$7.17 \times 10^{-4} sec^{-1}$	0.900	2×10^{-4}	
	$3.99 \times 10^{-4} sec^{-1}$	0.880	1×10^{-4}	4.44 ± 0.53 &mole^{-1}sec^{-1}
	$2.87 \times 10^{-4} sec^{-1}$	0.920	5×10^{-5}	
6% Brain Homogenate (37°C)	$2.51 \times 10^{-3} sec^{-1}$	0.995	1.22×10^{-4}	
	$1.99 \times 10^{-3} sec^{-1}$	0.997	9.79×10^{-5}	20.17 ± 0.24 &mole^{-1}sec^{-1}
	$9.59 \times 10^{-4} sec^{-1}$	0.995	4.89×10^{-5}	
40 or 80% Plasma (37°C)	$2.37 \times 10^{-4} sec^{-1}$	0.993	5×10^{-5}	
30% Liver Homogenate (37°C)	$3.74 \times 10^{-4} sec^{-1}$	0.995	5×10^{-5}	

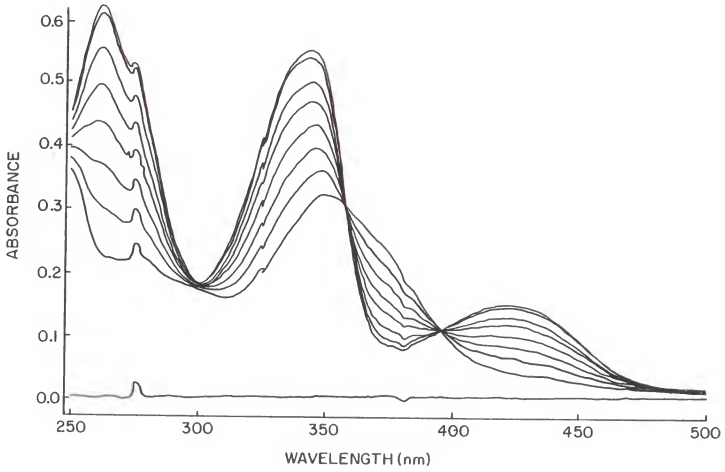


Figure 3-14. Spectral Changes of Dihydroberberine (2) upon Oxidation to Berberine (1) in pH 5.8 Phosphate Buffer at 26°C. Traces were made every 10 min.

ℓ/mole sec. A number of oxidants were investigated to study the oxidation of (2). Unfortunately, these produced rates of oxidation which were far too rapid to analyze by simple means.

The rate of oxidation of (2) in various biological media was also determined and these results are presented in Table 3-13. Second-order kinetics were again observed. In brain homogenates the correlation coefficients were much higher than those obtained in buffer. In the majority of the determinations, pseudo first-order rate constants at specific concentrations were calculated. The values obtained allow a comparison from system to system. The $t_{\frac{1}{2}}$, for example, of oxidation of a 5×10^{-5} M solution of (2) in plasma, liver homogenate, and brain homogenate was calculated to be forty-eight minutes, thirty-one minutes, and approximately twenty-five minutes, respectively. The oxidation of (2) by a second-order process is very characteristic of many dihydropyridines.

The relative stabilities of dihydropyridines were investigated by comparing their rates of oxidation in dilute hydrogen peroxide. This system was reproducible and, as shown in Table 3-14, gave data of good quality. The $t_{\frac{1}{2}}$ calculated from the pseudo first-order rate constants obtained at 1×10^{-4} M for (2), 1-methyl-1,4-dihydronicotinamide (21), and 1-benzyl-1,4-dihydronicotinamide (22) were 25.6 min, 1.2 min, and 11.5 min, respectively. These results are consistent with the greater stabilization of (2) compared to simpler systems, which was predicted by the theoretical calculations. The rate constants are relatively small because of the slightly acidic nature of the peroxide. At the end of the experiment, (1) was analyzed to make sure that nucleophilic addition of the peroxide to (2) did not occur.

The Mechanism of Oxidation of Dihydroberberine

A knowledge of the mechanism of oxidation of dihydropyridine could be helpful in applying the drug delivery system. The mechanism of oxidation

Table 3-14. The Relative Rates of Oxidation of Dihydroberberine (2), 1-Methyl-1,4-dihydronicotinamide (21), and 1-Benzyl-1,4-dihydronicotinamide (22) in Dilute Hydrogen Peroxide

Compound	Pseudo First Order Rate Constant	Correlation	Relative Rate of Oxidation
(2)	4.51×10^{-4}	0.998	1.0
(21)	9.83×10^{-3}	0.99999	21.7
(22)	1.00×10^{-3}	0.999	2.2

of simple dihydropyridines, particularly dihydronicotinamides, has been extensively studied since these partial structures occur in the $NADH \rightleftarrows NAD^+$ system. Many models of this system have been used and most are simple, substituted 1,4-dihydronicotinamides.

In the classic work of Abeles and Westheimer, the oxidation of substituted 1,4-dihydronicotinamides by thiobenzophenones was studied.[194] Like most dihydropyridines, these exhibit second-order kinetics: first-order with respect to the dihydropyridine and first-order with respect to the thiobenzophenone. The mechanism of oxidation proposed by this group was that of a concerted hydride transfer from the dihydronicotinamide to the thiocarbonyl carbon. This mechanism has been modified over the years.[195-197] Most recently, Ohno has described a system in which the oxidation proceeds through a charge transfer complex.[198,199] The initial step in this process is an electron transfer, followed by a proton transfer followed, in turn, by a subsequent electron transfer. In free radical oxidations Eisner, using substituted 1,4-dihydronicotinamides and the oxidant, diphenylpicrylhydrazyl free radical (DPP·), again found a second-order oxidative process with the rate-determining step being the initial abstraction of a hydrogen.[200] This is followed mechanistically by the formation of the quaternary compound. The oxidation of other dihydropyridines, such as the free radical oxidation of dihydroanthracene or dihydrophenanthrene, has also been reported.[201,202] The kinetics of enzymatic oxidation of dihydronicotinamides have also been studied. In 1980 Porter and Bright published an article on the oxidation of substituted 1,4-dihydronicotinamides by lumiflavins and old yellow enzyme.[203] Kinetically, a second-order oxidation was found to take place, mediated by a charge transfer or biradical complex.

The mechanism of oxidation of dihydropyridines is, therefore, dependent on the oxidant and the conditions under which oxidation takes place. A series of experiments was performed to investigate the mechanism of oxidation of simple dihydronicotinates and (2), and to determine if the oxidation of the dihydropyridines is mediated by an enzyme or by some other species such as dissolved oxygen.

In these experiments a homologous series of 1-methyl-1,4-dihydronicotinic acid esters was synthesized. The NMR of a representative compound is presented in Figure 3-15, and the corresponding proton assignments in Table 3-15. The rate of oxidation of (22), (23), (24), (25), (26), (27), and (28) in 40% human plasma, 6% brain homogenate, or 3.5% liver homogenate was measured. This was done by determining the rate of disappearance of the 359 nm absorption of the dihydronicotinamide with time. Since the rate of ester hydrolysis is slower for nicotinic acid esters and much slower for 1-methylnicotinic acid esters than the values obtained, the results clearly represent the oxidation process of the dihydropyridine and not hydrolysis. In all determinations the concentration of the dihydropyridines was 5×10^{-5} M. The results of this experiment are shown in Figure 3-16 and Table 3-16. Both in plasma and in buffer, the rate of oxidation as measured by the pseudo first-order rate constant, is relatively slow and the correlation coefficients are relatively small. There is also little effect on the rate constants by the molecular structure. This indicates a nonspecific oxidative route. In organ homogenates, there is a marked acceleration in the rate of oxidation as well as an increase in the correlation coefficients. There is also a large dependence upon the rate by the structure of the molecule and, in general, as the chain length increases, the rate decreases. These three changes - acceleration of the rate, linearization of the data, and the greater reliance of the

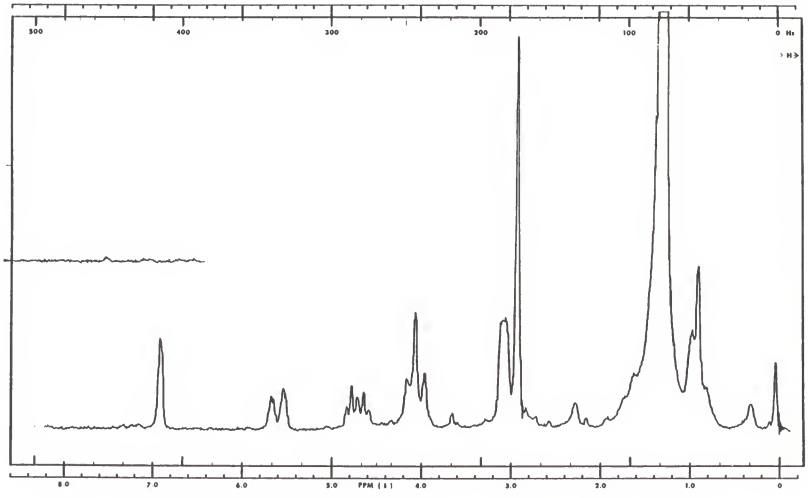
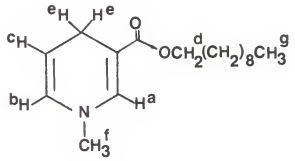


Figure 3-15. Proton Nuclear Magnetic Resonance Spectrum (60 MHz) of (27) in $CDCl_3$

Table 3-15. Proton Assignments of the ^1H NMR of the 1-Methyl-1,4-dihydronicotinic Acid Ester (27)



Proton	PPM (δ)
a	6.9
b	5.6
c	4.7
d	4.0
e	3.0
f	2.9
g	0.9

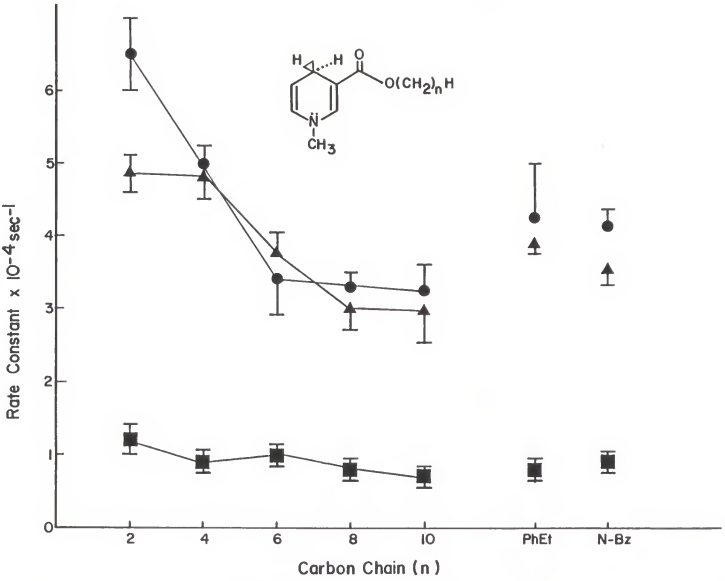


Figure 3-16. The Rates of Oxidation of Various 1-Methyl-1,4-dihydronicotinic Acid Esters (23), (24), (25), (26), (27), (28) and 1-Benzyl-1,4-Dihydronicotinamide (22) at 37°C in 40% Human Plasma (■), 6% Brain Homogenate (▲) and 3.5% Liver Homogenate (●)

Table 3-16. The Rates of Oxidation and Corresponding Correlation Coefficients of Various 1-Methyl-1,4-dihydronicotinic Acid Esters and 1-Benzyl-1,4-dihydronicotinamide (22)

Compound Number	Pseudo First Order Rate Constants x $10^{-4}sec^{-1}$					
	Plasma	Correlation	Brain Homogenate	Correlation	Liver Homogenate	Correlation
(23)	1.19 ± 0.14	.970	4.87 ± 0.25	.9998	6.49 ± 0.51	.998
(24)	0.90 ± 0.11	.921	4.80 ± 0.27	.9998	4.97 ± 0.19	.998
(25)	0.97 ± 0.10	.962	3.75 ± 0.31	.9998	3.40 ± 0.55	.981
(26)	0.81 ± 0.03	.971	3.08 ± 0.33	.998	3.29 ± 0.16	.974
(27)	0.71 ± 0.05	.940	3.02 ± 0.44	.9991	3.25 ± 0.39	.993
(28)	0.82 ± 0.06	.976	3.88 ± 0.09	.9994	4.25 ± 0.91	.998
(22)	0.90 ± 0.05	.990	3.53 ± 0.17	.9992	4.14 ± 0.16	.9990

rate on structure - indicate the involvement of an enzyme in the oxidation. One of the enzymes which is said to be responsible for the oxidation of dihydropyridines is NADH dehydrogenase.[204] Since this family of enzymes is membrane bound, it would be present in the organ homogenate but not in plasma. The results are consistent with this distribution. Enzymes involved in the oxidation of dihydropyridines have as their endogenous substrate NADH. This molecule is not substituted at the amide nitrogen and one would expect compounds which more closely resemble NADH to be better substrates for the enzyme than molecules which greatly deviate from this structure. One would predict that the shorter chain analogs (23) and (24), and the N-benzyl compound (22) would be oxidized more rapidly than the longer chain analogs and this is, in fact, the case. An additional observation which supports this hypothesis is that as the homogenates age, the rate constants as well as the correlation coefficient decreases. This is consistent with the time-dependent denaturation characteristic of this type of enzymatic system.

These trends also occur in the oxidation of (2). In plasma and buffer, the data indicates a relatively slow oxidation with poor correlation. In brain homogenate, there is an acceleration and a linearization in the rate constants. These results indicate that although oxidation of (2) can be mediated by oxygen, in tissues like the brain and liver, an enzymatic oxidation can occur. The effect of protein binding on the rate of oxidation of (2) was investigated by changing the concentrations of the homogenates. One would expect (2) to bind to proteins but the effects of this complexation on oxidation were not large.

Membrane Permeability of Dihydroberberine and Berberine

In order to investigate the relative ability of dihydroberberine (2) to penetrate membranes, the behavior of (2) and (1) in a model system was

observed. The model system which was chosen in this study was that of the red blood cell. In this experiment (1) or (2) were placed in a known volume of whole blood, and at various times the concentration was determined in the plasma or packed red blood cells. The results are shown in Figure 3-17. As one can see, the initial rate of penetration of (2) into red blood cells is rapid and greater than that of (1). The initial concentration is also higher. This affinity for the red blood cells is mirrored by a disappearance of (2) from the plasma. With time, equilibration occurs in the system. Berberine penetrates the red blood cell slowly and reaches a maximum concentration much later than does (2). This is another indication of the increased membrane mobility of (2) relative to (1). The two curves finally converge to the same value, as the oxidation of (2) to berberine takes place. Creasey indicated that there was a relationship between glucose transport and the transport of berberine into red blood cells.[143] To investigate this possibility, the behavior of berberine in a red blood cell system which contained 200 mg% glucose was observed. As shown in Table 3-17, there is very little effect of glucose on the entry of berberine into the red blood cells from the plasma.

In Vivo Studies

The preliminary studies indicate that dihydroberberine possesses all of those characteristics required of a compound which is to be applied to the drug delivery system proposed in Figure 1-3. The substantiation of this drug delivery scheme requires not only the demonstration of the delivery of (1) after administration of (2) but also the specific retention of (1) in the brain. The first priority of the in vivo system was to show delivery into the brain of (1). The protocol used in these studies involved injecting rats with either berberine (1), dihydroberberine (2), or its hydrochloride (3). After a period of time the chest cavities of

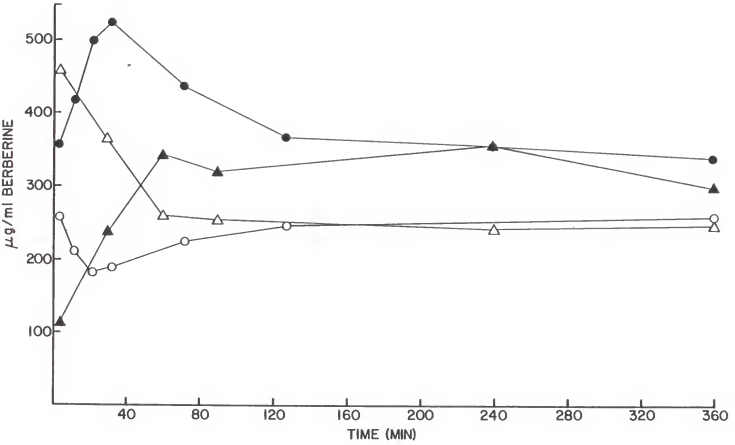


Figure 3-17. Partitioning of 26.5 mg of Berberine (1) from Plasma (△) into Red Blood Cells (▲) and of 26.5 mg of Dihydroberberine Hydrochloride (3) from Plasma (○) into Red Blood Cells (●). The Volume of Blood Used in each Experiment was 75 ml

Table 3-17. The Effect of Glucose on the Movement of Berberine into Red Blood Cells

Time (min)	Berberine Concentration (μg/ml) Plasma[a]	
	Control	+ 200 mg% Glucose
5	360.94	352.29
30	300.39	309.04
60	274.95	276.48
90	267.29	255.62
120	259.17	272.92

[a]This is a representative experiment selected from a group of three

the animals were opened, the heart perfused with saline, and the brain removed. High pressure liquid chromatography was used in analysis.

The initial experiments showed that after administration of 55 mg/Kg of (1) in dimethylsulfoxide (DMSO), no (1) could be detected in the brain at any time. When, however, 55 mg/Kg of (2) in DMSO were administered, a high concentration of (1) was observed in the brain as shown in Figure 3-18. The free base was not exceptionally stable in solution, however, and was also very water insoluble. For these reasons the hydrochloride (3) was prepared and used in subsequent experiments. If 55 mg/Kg of (3) in 20% aqueous ethanol are injected systemically, the concentration of (1) in the brain is again found to be relatively high. This is presented in Figure 3-19. The concentration of (1) achieved in the brain after administration of (2) or (3) was similar (approximately 50 μg/g tissue). The loss of (1) from the brain after administration of (3) is slow and the $t_{\frac{1}{2}}$ of this loss, calculated from the terminal portion of the log concentration versus time relation, is approximately eleven hours.

It should be emphasized that in these experiments only the concentration of (1) was measured even though unoxidized (2) was present. To obtain a more complete picture of the behavior of (1) in the brain, the total berberine concentration, i.e. (1) and (2) was measured and this appears in Figure 3-20. The concentration of (2) is high at early time points but diminishes rapidly. The initial rate of disappearance of (2), obtained by subtracting the concentration of (1) in the brain from the total concentration (Figure 3-21), yields a $t_{\frac{1}{2}}$ of thirty-four minutes which is of the same magnitude as the value obtained from brain homogenates. The slope of the terminal portion of the curve in Figure 3-20 yields a $t_{\frac{1}{2}}$ of 5.7 hours.

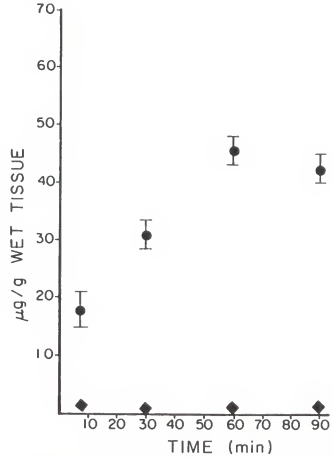


Figure 3-18. Distribution of Berberine in the Brain after iv Administration of Berberine (1) (◆) at a Dose of 55 mg/Kg or of Dihydroberberine Free Base (2) (●) at a Dose of 55 mg/Kg

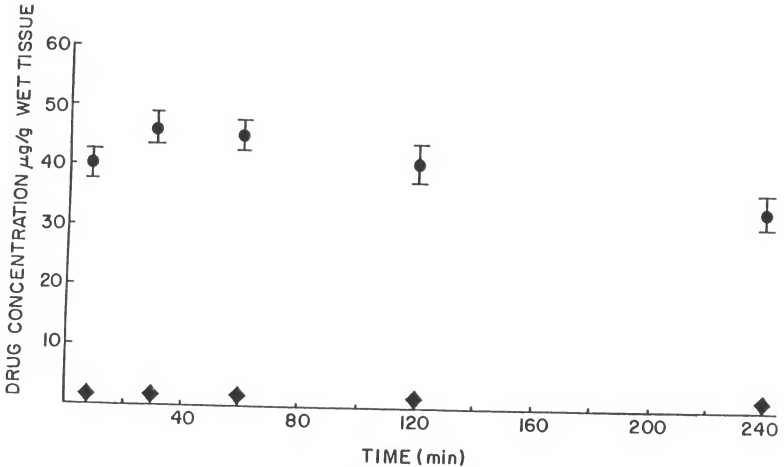


Figure 3-19. Efflux of Berberine from the Brain after iv Administration of either 55 mg/Kg of Dihydroberberine Hydrochloride (3) (●) or 55 mg/Kg of Berberine (1) (◆). Analysis was for (1) only and not Unoxidized (2)

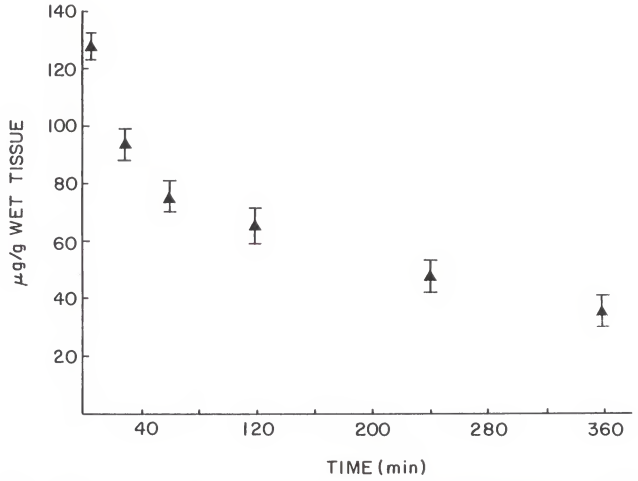


Figure 3-20. Efflux of Berberine (1) and Unoxidized Dihydroberberine (2) (▲) after i v Administration of 55 mg/Kg of Dihydroberberine Hydrochloride (3)

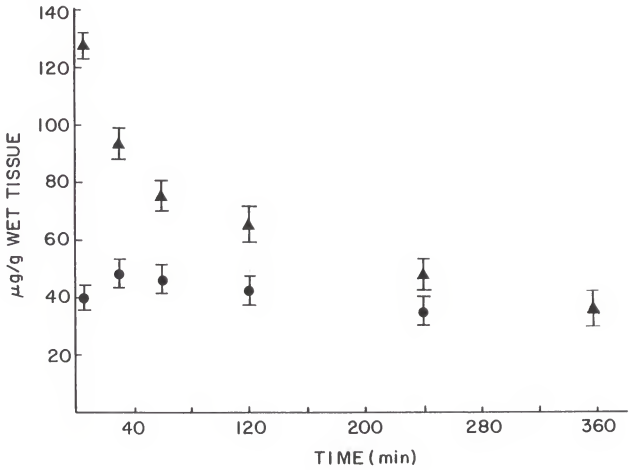


Figure 3-21. A Comparison of the Efflux of Berberine (1) (●) and Berberine (1) and Unoxidized Dihydroberberine (2) (▲) after a Dose of 55 mg/Kg of Dihydroberberine Hydrochloride (3) Administered i v

This indicates a faster efflux for the total berberine concentration than for the efflux of (1) alone. The reason for this is that, aside from efflux of (1) from the brain, a component of the oxidation of (2) and for the efflux of (2) also occurs in this rate. Since the oxidation of (2) is not immediate, a significant quantity of it is present at later times and this population of molecules will redistribute out of the brain as a function of the blood concentration of (2) as would any lipophilic compound. This added equilibrium complicates the kinetics of the original scheme.

The distribution of berberine (1) in various tissues after an intravenous dose of 35 mg/Kg is shown in Figure 3-22. The lower dose was used because of the higher toxicity of (1). These results show a high concentration of (1) in the kidney and, to reiterate, no quantitatable amount in the brain. Berberine is rapidly lost from the tissue and this contributes to its relatively short biological $t_{\frac{1}{2}}$. The distribution of (1) in the tissues has been previously studied.[205-209] The results obtained from a number of these studies are consistent with those reported here. Berberine is rapidly lost from the tissues and effectively excreted by the kidney and by a biliary route. In literature reports the concentration of (1) in the brain was always undetectable or the lowest of all other tissues examined. In those cases where (1) was detected, the amounts found were usually on the order of 50-200 ng/g tissue which is below the limit of detection in this study. These values are low in both relative and absolute terms. Since the effective concentration of (1) in in vitro systems is on the order of 1.0 μg/g, these levels would be ineffective therapeutically. These studies also indicate berberine is not absorbed to a great extent from the intestine, again reflecting its highly polar nature.

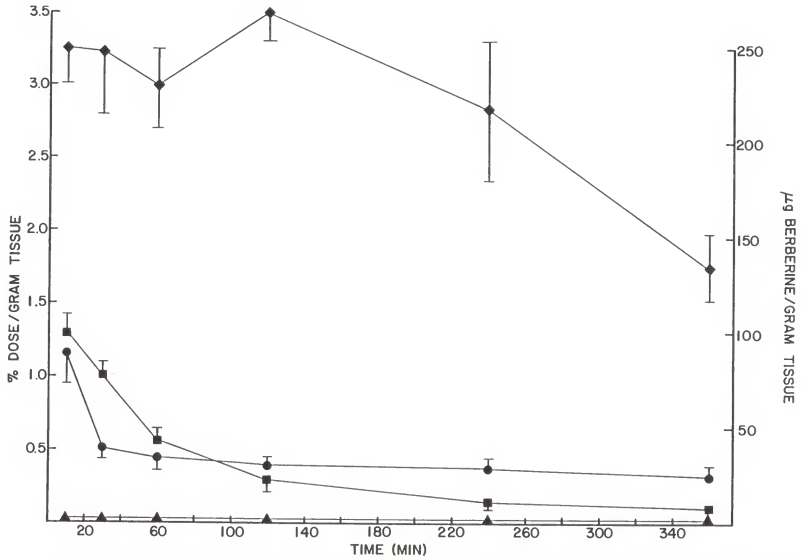


Figure 3-22. Distribution of Berberine after i v Administration of 35 mg/Kg of Berberine (1) into the Kidney (◆), Liver (■), Lung (●), and Brain (▲)

The tissue distribution of dihydroberberine hydrochloride (3) was investigated in an analogous manner. The compound (3) is taken up by tissues to a greater extent than (1) and is handled well by the kidney. The concentration of (1) observed in the brain is high. A comparison of the time courses of (1) after administration of either (1) or (3), in various organs appears in Figures 3-24 to 3-26. These results shown are consistent with the greater ability of (3) to penetrate tissues. The liver (Figure 3-26) is noteworthy because (1) is known to be significantly excreted by a biliary mechanism.

In order to better illustrate the specificity of the (1) $\rightleftarrows$ (2) system, (3) was infused intravenously. The results of these infusions are presented in Table 3-18. In these administrations the standard dose of 55 mg/Kg of (3) was given over a period of either thirty or forty-five minutes. If a comparison is made between the concentration of (1) in various organs at the end of the infusion and the concentration of (1) obtained at thirty or forty-five minutes after a bolus iv injection, the specificity inherent in the system can be demonstrated. If the system is not specific, one would expect to see an overall decrease in the concentration of (1) after an iv infusion of (3) compared to the bolus. The results show that at thirty minutes, there is a higher concentration of (1) in the brain compared to the iv bolus and a decrease in the concentration of (1) in the lungs and kidneys. The liver shows a moderate increase. At forty-five minutes these trends continue. A comparison of the data at thirty and forty-five minutes shows an increase of the concentration of (1) in the brain and a reduction in all other organs. The concentration of (1) in the brain is higher than in any other tissue analyzed at forty-five minutes.

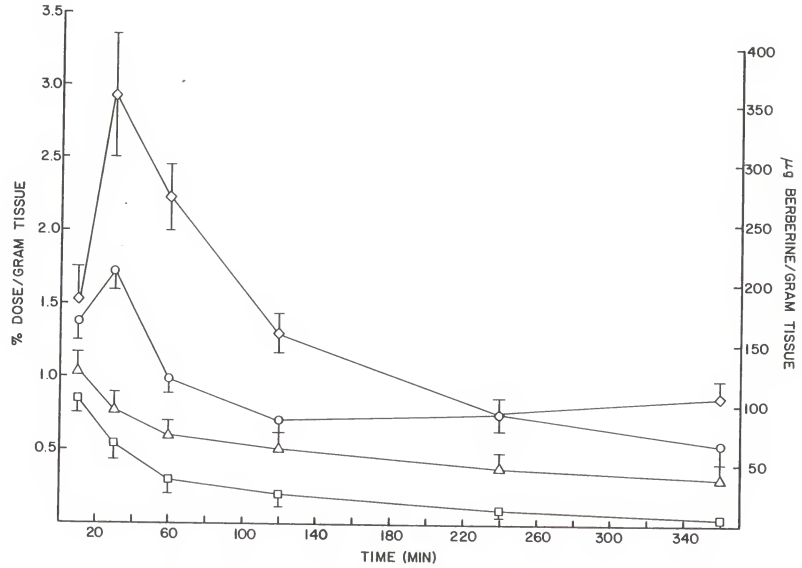


Figure 3-23. Distribution of Berberine after i v Administration of 55 mg/Kg of Dihydroberberine Hydrochloride (3) into the Kidney (◇), Liver (□), Lung (○), and Brain (△)

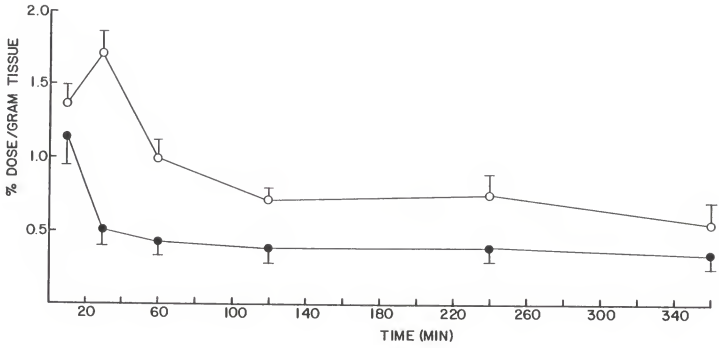


Figure 3-24. A Comparison of the Efflux of Berberine from Lungs when Administered i v as 55 mg/Kg of Dihydroberberine Hydrochloride (3) (O) or 35 mg/Kg of Berberine (1) (●)

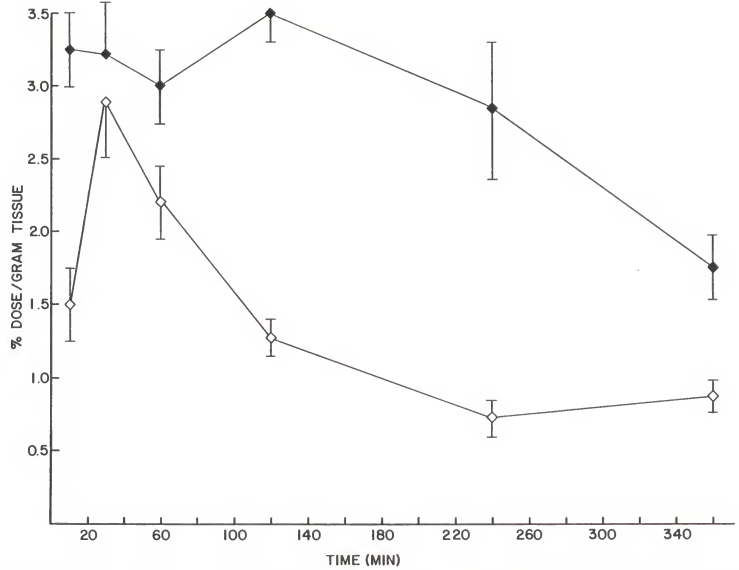


Figure 3-25. A Comparison of the Efflux of Berberine from the Kidneys when Berberine (1) is Administered i v at a Dose of 35 mg/Kg (◆) and Dihydroberberine Hydrochloride (3) when Administered i v at a Dose of 55 mg/Kg (◇)

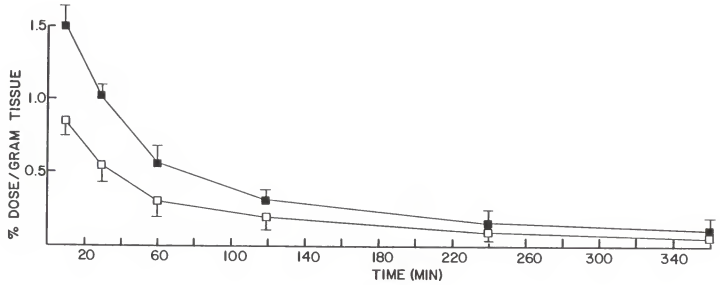


Figure 3-26. A Comparison of the Efflux of Berberine from the Liver when Berberine (1) is Administered i v at a Dose of 35 mg/Kg (■) and Dihydroberberine Hydrochloride (3) when Administered i v at a Dose of 55 mg/Kg (□)

Table 3-18. Slow Infusion of Dihydroberberine (3)

Organ	30 min Concentration (μg/g) after infusion	Concentration (μg/g) 30 min after iv bolus	Δ(μg/g)
Brain	135.95 ± 13	91.8 ± 20	+ 44
Kidney	185.5 ± 26	351.8 ± 54	-166
Lung	71.4 ± 10	210.2 ± 14	-139
Liver	101.2 ± 23	67.8 ± 11	+ 33

Organ	45 min Concentration (μg/g) after infusion	Concentration (μg/g) 45 min after iv bolus	Δ
Brain	162.2 ± 8	88	+ 74
Kidney	121.4 ± 19	315	-194
Lung	62.8 ± 6	165	-102
Liver	79.4 ± 10	52	+ 27

These data verify the drug delivery scheme devised. After administration of (3) high concentrations of (1) are obtained specifically in the brain, while the concentrations in other organs are reduced. The reason that the slow infusion of (3) enhances the specificity is related to a number of factors. Dihydroberberine is a lipophilic compound. The iv bolus injection presents to the tissues a large concentration of (2) and the result of this large burden is an inordinately long transit time of (2) in peripheral sites. This obfuscates the kinetics. By slowly administering (3), the tissue burden is greatly reduced, and the designed improvements in the bidirectional characteristics of the delivery molecule can be fully demonstrated. This system in general, and (2) in particular, allows specific delivery of quaternary compounds to the brain. This system is designed to reduce systemic levels of an agent and thereby reduce any accompanying toxicity.

Efflux of Berberine from the Brain

The mechanism by which berberine leaves the brain is not known but it is important to the quaternary scheme. According to the original postulation, large quaternary compounds like (2) should leave the brain slowly, presumably by passive processes such as movement in the CSF. Small quaternary compounds, on the other hand, are substrates for active carriers which rapidly remove these compounds from the brain extracellular fluid.[45] The next series of experiments was designed to determine the mechanism of efflux of (1) from the brain. If the efflux of (2) is mediated by a specific carrier, it should be possible to demonstrate the competitive inhibition of the efflux of (2) by introducing into the system a large concentration of another agent which has affinity for the cationic pump, such as 1-methylnicotinamide (6) and 1-benzylnicotinamide (7).

The first experiments involved injecting rats with 200 mg/Kg of 1-methyl-1,4-dihydronicotinamide (21) in aqueous ethanol followed fifteen minutes later by an injection of the standard dose of 55 mg/Kg of (3). The results of this study are shown in Figure 3-27. The dihydronicotinamide (21) was used as a proform of quaternary compound (6) in an attempt to generate high levels of (6) in the brain. The results do not show, however, any significant difference in the efflux of berberine (1) between pretreated and unpretreated animals.

In an analogous study 1-benzylnicotinamide (7) and its corresponding dihydro adduct (22) was employed. Unlike the 1-methyl derivatives, this pair of compounds possesses a UV chromophore which greatly simplifies quantitation. A preliminary study was performed to determine the time at which the maximum concentration (t_{max}) of (7) after the administration of 1-benzyl-1,4-dihydronicotinamide (22) occurred. The results of this experiment are shown in Figure 3-28. After injection of (7) no detectable levels of (7) in the brain could be observed. The reason the t_{max} is important is that, ideally, the lag time between the injection of the antagonist, i.e. (7) and (2) is the time required for (7) to reach a maximum concentration. Unfortunately, however, the toxicity of dihydrobenzyl derivative (22) proved to be much higher than that of dihydromethyl derivative (21) and the maximum dose which could be given was only 60 mg/Kg and, because of this, further studies with this compound were abandoned.

A number of observations concerning this figure are germane to the topic of the efflux of (1) from the brain. In the case of administration of this N-benzyldihydropyridine (22), no dihydronicotinamide is present at early time points at the dose used in the experiment. This is not the case with (2). This is consistent with the greater stability of (31) and hence, (2), predicted by the theoretical calculations. The $t_{\frac{1}{2}}$ of efflux

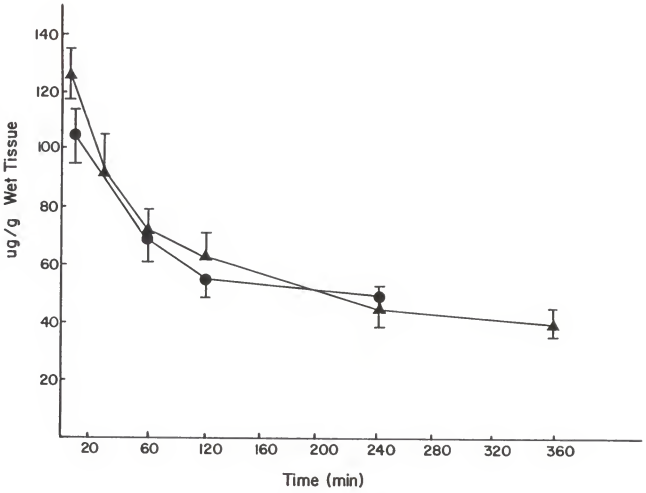


Figure 3-27. A Comparison of the Efflux of the Total Berberine, i.e. (1) and (2) from the Brain when either 55 mg/Kg of Dihydroberberine Hydrochloride (3) is Administered i v (▲) or 55 mg/Kg of Dihydroberberine (2) and 200 mg/Kg of 1-Methyl-1,4-dihydronicotinamide (21) is Administered i v (●)

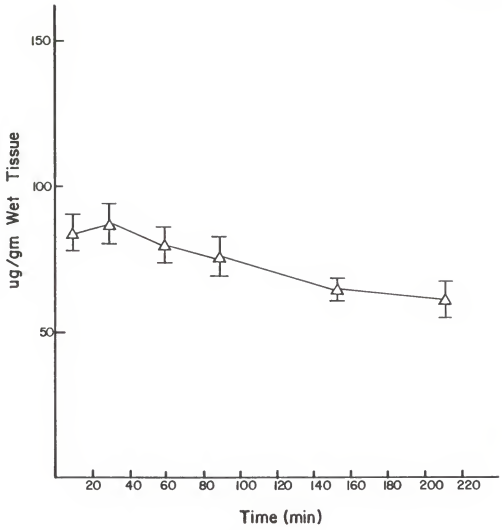


Figure 3-28. Efflux of 1-Benzylnicotinamide Bromide (7) from the Brain after i v Administration of 60 mg/Kg of 1-Benzyl-1,4-dihydronicotinamide (22)(△)

from the N-benzyl quaternary compound (7) from the brain is 5.8 hours, which is twice as fast as the efflux of (1).

These studies indicate that there was no effect of the quaternary compound N-methylnicotinamide (6) on the efflux of (1) from the brain. The concentration difference, however, between (6) and (2) was only a factor of four at the injection, and this difference is even less at the level of the brain. To overcome this quantitative objection, a series of intracerebral ventricular (icv) injections was performed. The purpose of injecting the compound icv was to allow direct introduction of high concentrations of (1) and (1) with the putative competitive inhibitor, (6), into the brain. The injections were made into the lateral ventricles of rats with the aid of a stereotaxic instrument and an infusion pump. A dose of 50 μg of (1) or 50 μg of (1) and 1000 μg of (6) was administered and the compounds were dissolved in DMSO. The volume of the dose was 3-5 μl. The results of these studies are shown in Figure 3-29. It can be seen that (6) has little effect on the efflux of (1) from the brain. The $t_{\frac{1}{2}}$ obtained from the terminal portion of the curves was 3.8 hours for the efflux of (1) and 3.1 hours for the efflux of (1) when coinjected with (6). The fact that the $t_{\frac{1}{2}}$ obtained for (1) in this experiment is much slower than that obtained when (1) is administered systemically as (3) is not surprising. These icv injections are similar to intrathecal injections in that even or complete distribution does not occur. Therefore, (1) is basically restricted to the CSF. In the case of systemic administration of (3), however, the distribution of (2) is fairly complete and even in the brain. The efflux of (1) after administration of (3) is limited by the diffusion of (1) from the brain tissue, and from the discussion of the BBB, this is a slow and inefficient process. When (1) is administered icv,

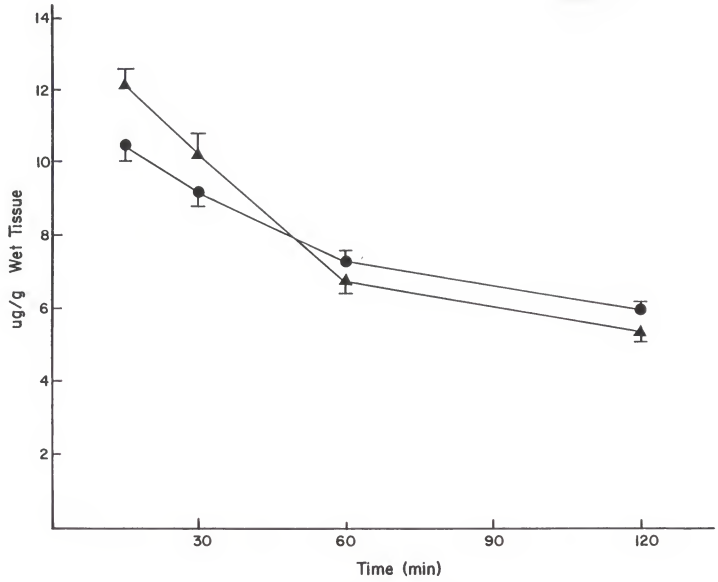


Figure 3-29. Efflux of Berberine from the Brain after icv Injection of either 50 μg of Berberine (1) (●) or 50 μg of Berberine (1) and 1000 μg of 1-Methylnicotinamide Iodide (6) (▲)

the limitations of diffusion do not apply and (1) may simply be lost by the bulk flow of CSF.

To determine the rate of CSF flow in the animal system used in these experiments, the rate of efflux of ^{3}H-inulin (29) from the brain was measured. This radionuclide was injected icv in DMSO at a dose of 2.3 μCi. As shown in Table 3-19, the $t_{\frac{1}{2}}$ of efflux of (29) is two hours, in good agreement with previously reported values.[45]

The loss of (1) from the brain appears not to be mediated by an active process. Simple bulk flow of CSF seems to be sufficient to remove (1) from the brain. The rate-limiting step in the cerebral elimination of (1) does not appear to be the actual efflux process but rather, the redistribution of (1) out of brain cells and membranes by this poorly mobile species to those areas where elimination can take place.

Limited Metabolic Studies

The metabolism of (1) after the administration of (1) or (3) was studied in the rat. Urine collected for three days after a dosing of (1) or (3) was extracted with chloroform or 3-methyl-1-butanol. The alcohol was used because it is reported to extract berberine and similar alkaloids efficiently from aqueous solutions.[210] The results of the HPLC and TLC analyses are shown in Tables 3-20 and 3-21, respectively. As one can see, there is very little metabolism of (1) and the major component of the urine from animals dosed with either (1) or (3) was (1). There were several peaks which could not be attributed to the anesthetic and these were found in the urine of all tested animals. The size and retention time of these peaks was again similar.

These data are consistent with the few articles which have been published concerning the metabolism of (1). While this metabolism is reported

Table 3-19. Efflux of ^{3}H-Inulin from the Brain after Intracerebral Ventricular Administration

Time (min)	% Dose/g Wet Tissue	In % Dose/g Wet Tissue
15	9.28%	2.23
30	7.94%	2.07
45	6.52%	1.87
60	5.54%	1.71
120	4.71%	1.55
Corr. = 0.930	$k = 6.23 \times 10^{-3} min^{-1}$	$t_{\frac{1}{2}} = 1.9$ hrs

Table 3-20. In Vivo Metabolism of Berberine and Dihydroberberine in the Rat (HPLC)

HPLC[a] Compound	Retention time of Peaks Observed (min)	Peak Height Relative to Berberine
Berberine		
Chloroform Extract	1.8[X]	0.73
	2.0[X]	0.44
	2.5[X]	0.39
	2.9	0.05
	4.2[*]	1.0
	5.6	0.01
Isoamyl Alcohol Extract	1.7[X]	4.7
	2.5[X]	1.4
	3.6	0.28
	4.0[*]	1.0
	4.8	0.09
Dihydroberberine		
Chloroform Extract	1.8[X]	0.52
	2.1[X]	0.37
	2.6[X]	0.35
	3.0	0.04
	4.2[*]	1.0
	5.6	0.01

Table 3-20-continued.

HPLC Compound	Retention Time of Peaks Observed (min)	Peak Height Relative to Berberine
Dihydroberberine		
Isoamyl Alcohol Extract	1.9[X]	5.1
	2.5[X]	1.3
	3.4	0.18
	4.1*	1.0
	4.8	0.09

*Corresponds to berberine standard

[X]These peaks were found in blank animals injected with the anesthetic

[a]Five microliters of the sample were injected on a μBondapak C_{18} reverse phase column. The mobile phase was acetonitrile:pH 6.2 phosphate buffer 60:40 and the flow rate was 2.0 ml/min

Table 3-21. In Vivo Metabolism of Berberine and Dihydroberberine in the Rat (TLC)

TLC[a] Compound	R_f of Spots Observed
Berberine	
Chloroform Extract	0.15*
	0.125[X]
	0.21[X]
	0.34
Isoamyl Alcohol Extract	0.14*
	0.34
Dihydroberberine	
Chloroform Extract	0.21*
	0.35
Isoamyl Alcohol Extract	0.21*
	0.26[X]
	0.29
	0.60

*Corresponds to berberine standard

[X]These spots were found in blank animals injected with the anesthetic

[a]Five microliters of the sample were spotted on alumina plates and eluted with cyclohexane:chloroform:acetic acid 45:45:10

be to minimal in vivo, two minor metabolites have been identified. Furuya described a urinary metabolite which apparently contained a carboxylic acid moiety.[211] Another study reported that very small amounts of tetrahydroberberine were present in the urine.[208] Identification of the metabolites found in the present study was not attempted.

The importance of these studies to the proposed drug delivery scheme is related to the requirement of the scheme that the principal and, ideally, only metabolism of (2) is to (1). This was shown to be the case. In addition, no metabolite was present in the urine extracts of animals dosed with (3) which was not present in the urine extracts of animals dosed with (1).

Toxicity and Anticancer Activity of Dihydroberberine

The toxicity of (1) and (3) was determined in mice and the results are shown in Figure 3-30 and Table 3-22. The data were analyzed by the method of Probits as well as by fitting the data to a sigmoid dose-response curve. The lethal dose for 50% mortality (LD_{50}) for (1) was found to be 37.0 mg/Kg, and that of (3), 58.2 mg/Kg. The injections were made intraperitoneally. The value obtained for (1) was in good agreement with other values reported in the literature.[144,212] The toxicity of (1) is 60% higher than that of (3), substantiating a prediction made by the theoretical calculations. The anticancer activity of (1) and (3) is presented in Table 3-23. First, the ability of (1) or (3) to inhibit the growth of KB cells in vitro was investigated. The ID_{50} calculated for (3) was 2.2 μg/ml while that calculated for (1) was 0.95 μg/ml. This is again constant with the higher toxicity of (1) relative to (3).

The next series of experiments involved inoculating mice with P388 lymphocytic leukemia cells. If this is done ip, (1) and (3) are equipotent

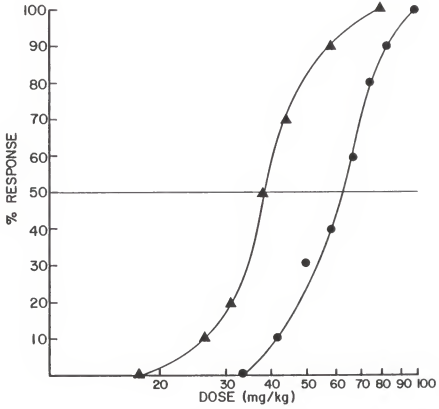


Figure 3-30. The LD_{50} Dose-response Curve of Berberine (1) (▲) and Dihydroberberine Hydrochloride (3) (●). Doses of (1) or (2) were Administered i p in CD-1 Mice

Table 3-22. Probit Analysis of the LD_{50} Study

	Dose mg/Kg	In [Dose]	% Dead	Probit
Berberine	10	2.303	0	——
	17	2.833	0	——
	24	3.178	10	3.718
	31	3.434	20	4.150
	38	3.637	50	5.000
	45	3.807	70	5.524
	59	4.078	90	6.282
	80	4.382	100	8.719
Dihydroberberine Hydrochloride	25	3.219	0	——
	33.3	3.506	0	——
	41.6	3.728	10	3.718
	50	3.912	30	4.476
	58.3	4.066	40	4.747
	66.6	4.199	60	5.253
	75	4.317	80	5.842
	83.3	4.422	90	6.282
	100	4.605	100	8.719

Table 3-23. Effect of Berberine (1) and Dihydroberberine Hydrochloride (3) Against P388 Lymphocytic Leukemia

Drug	Dose mg/Kg	Number of mice	P388 i.p. Surv. Time (days)	P388 i.p. %ILS	P388 intracerebrally Surv. Time (days)	P388 intracerebrally %ILS
Berberine (1)	5	6	11.17 ± .40	10.8	9.83 ± .17	1.7
	10	6	11.33 ± .42	12.4	9.50 ± .22	-1.8
	20	6	10.50 ± .50	4.2	8.67 ± .56	-10.3
Dihydroberberine Hydrochloride (3)	5	6	10.83 ± .17	7.4	10.50 ± 1.31	8.6
	10	6	11.00 ± .30	9.1	10.00 ± .37	3.4
	20	6	11.17 ± .17	10.8	11.33 ± 1.63	17.2
Vehicle	5 (ml/Kg)	12	10.08 ± .15	___	9.67 ± .14	___

in increasing the life span (ILS) of the animals compared to a control group. However, when the leukemia is inoculated intracerebrally, (3) is significantly more effective in increasing life span than is (1). The % ILS actually falls when (1) is administered. This is indicative of the toxic peripheral effects of (1). These preliminary anticancer studies again support the original hypothesis. The dihydropyridine is capable of passing the BBB and oxidizing to (1) where it may exert its cytotoxic activity.

Conclusions

This dissertation has presented a broadly applicable drug delivery scheme which is specific for the brain. This delivery method is based on a dihydropyridine-pyridinium salt redox system and on the multifaceted nature of the BBB. There are two major aspects of this delivery scheme. In the first, which is a chemical delivery system, a pyridinium carrier is attached to a drug molecule. The second, and the one with which this dissertation has dealt, is a prodrug system in which the carrier moiety is an integral component of the molecule. This system is simpler than the first but since the molecule to be delivered must contain a pyridinium partial structure, it is less general. In both cases the basis of the brain specific delivery is related to the greater lipophilicity of dihydropyridines, the ease of their oxidation and subsequent elimination peripherally and the difficulty with which large pyridinium compounds leave the CNS.

In order to substantiate the proposed method, it was applied to a salient example, berberine (1). This anticancer alkaloid contains a pyridinium moiety which is reducible and whose product of reduction is stable. The physical and chemical properties of dihydroberberine (2) were examined. Its rate of oxidation was found to be rapid in a number of media but not as rapid as the rate of oxidation of simple dihydropyridines. Dihydroberberine

was shown to be more lipophilic than berberine (1) and also better able to penetrate biological membranes. In an attempt to delve into the basic chemistry of these relatively unstable compounds, a model system was developed for them and this was examined by a MINDO/3 approach. The results obtained from this study were consistent with experimental data and were extendable to the berberine (1) $\rightleftharpoons$ dihydroberberine (2) pair. In addition, several predictions were made concerning the biological activity of (2) relative to (1) and these were found to be valid.

Dihydroberberine (2) or its hydrochloride salt (3) was injected iv into rats and when the brains were analyzed, high levels of (1) were found. No berberine (1) was found in the brain after systemic administration of (1). The rate at which (1) left the brain after its delivery by (3) was slow and the $t_{\frac{1}{2}}$ of the efflux was eleven hours. If dihydroberberine hydrochloride (3) is slowly infused iv, the concentration of (1) rises in the brain with time but falls in all other organs tested. At forty-five minutes the concentration of (1) is highest in the brain. The efflux of berberine (1) from the CNS appears to be mediated by a passive process, perhaps the bulk flow of CSF.

Dihydroberberine hydrochloride (3) was shown to be less toxic than (1) *in vivo* in accordance with predictions made by the theoretical studies. Additionally, (3) was shown to be less effective than (1) in inhibiting the growth of KB cells *in vitro*. While the two compounds are equipotent in increasing the life span of mice injected ip with a suspension of P388 lymphocytic leukemia cell, (3) is more potent in increasing the life span of mice who were inoculated intracerebrally with the P388 cell line.

These data verify the proposed drug delivery scheme. By concentrating a pharmacologically active agent at its site of action and by reducing its concentration in other locations, the therapeutic index of the delivered

compound is greatly enhanced. This was demonstrated when the delivery scheme was applied to the berberine (1)⇄dihydroberberine (2) example.

This method is potentially extendable to any drug which contains a pyridinium moiety. A number of anticancer agents such as nitidine, coralyne, and fagaronine fit these criteria. Phenothiazines and β-blockers, among others, could also be modified in this way to attain specific delivery to the brain.

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BIOGRAPHICAL SKETCH

Marcus Eli Brewster III was born in Jacksonville, Florida, on October 14, 1957, the 891st anniversary of the Battle of Hastings. He graduated from S. Wolfson High School in 1975. He then enrolled at Mercer University in Macon, Georgia, where he earned his B.S. cum laude in 1978 with a major in biology and a minor in chemistry. At Mercer he was a member of Lambda Chi Alpha social fraternity, Beta Beta Beta biological honor society and Gamma Sigma Epsilon chemical fraternity. After a summer position as a historian of the War between the States, he entered graduate school at the University of Florida, College of Pharmacy. Four years later he was granted a Ph.D. He is a member of American Chemical Society, American Pharmaceutical Association, American Association for Advancement of Science, and Society for Applied Spectroscopy.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Nicholas S. Bodor, Chairman
Professor of Medicinal Chemistry

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Kenneth B. Sloan
Assistant Professor of
Medicinal Chemistry

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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Professor of Chemistry

This dissertation was submitted to the Graduate Faculty of the College of Pharmacy and to the Graduate Council, and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

August 1982

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